Lipid biosynthesis and the NLRP3 inflammasome activation

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Declarations

Declaration of Originality

I declare that the work in this thesis is my own, all else is appropriately referenced. Fluorescence microscopy, Caspase-1 activity *in vivo*, ASC speck oligomerisation assay, endosome purification and immunoblotting for TGN38 experiments were performed by Najd Aljadeed and Paras Anand.

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Stuart Leishman

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Abstract

Our understanding of the immune system has advanced considerably in recent years; however, each new discovery brings further questions. One major finding in innate immunity is the NLRP3 inflammasome, a multiprotein complex that initiates programmed cell death through both canonical (caspase-1 mediated) and non-canonical (caspase-11 mediated) pathways. Lipids play essential roles in cellular processes, including development and metabolism. Fatty acid synthase (FASN), a homodimeric enzyme, is critical for cell growth and metabolic homeostasis by converting malonyl-CoA and acetyl-CoA into palmitate, a saturated fatty acid that serves as a precursor for longer-chain lipids. Additionally, palmitate can modify proteins through palmitoylation, binding to cysteine residues in target proteins to ensure their proper localization and function. My research demonstrates that inhibiting FASN with small-molecule inhibitors significantly suppresses NLRP3 activity, preventing the cleavage of downstream caspase-1 and Gasdermin D, and blocking the maturation of the pro-inflammatory cytokine IL-1β. Furthermore, I discovered that FASN activity is required for the palmitoylation of NLRP3 at the Cys898 site, a modification critical for NLRP3 localization to the dispersed *trans*-Golgi network, the site of inflammasome assembly. Additionally, large-scale untargeted mass spectrometry was conducted to map the cellular landscape of inflammasome activation. This unprecedented snapshot revealed various lipid classes influenced by both the canonical and non-canonical pathways. These findings provide new insights into the lipid-dependent regulation of NLRP3 and uncover novel mechanisms of inflammasome activation.

Publications

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Abbreviations

25-HC: 25-hydroxycholesterol

2-BP: 2-Bromopalmitate

ABE: Acyl-biotin exchange assay

ACP: Acyl carrier protein

ASC: Inflammasome Adaptor Protein Apoptosis-Associated Speck-Like Protein Containing CARD

ATP: Adenosine triphosphate

AIM2: Absent in melanoma 2

ALR: AIM-2-like receptors

BRCC3: BRCA1/2-containg complex subunit 3

CAPS: Cryopyrin-associated periodic syndrome

CARD: Caspase activation and recruitment domain

CASR: Calcium-sensing receptors

Cav-1: Caveolin-1

CLR: C-type lectin receptors

DAMP: Danger-associated molecular pattern

DC: Dendritic cell

DNA: Deoxyribonucleic acid

dTGN: Dispersed *trans* Golgi network

E.coli: *Escherichia coli*

ER: Endoplasmic reticulum

FASN: Fatty acid synthase

GAPDH: Glyceraldehyde 3-phosphate dehydrogenase

GSDMD: Gasdermin D

HA: Hydroxylamine

HMG-CoA: 3-hydroxy-3-methylglutaryl-CoA

IFN: Interferon

IL: Interleukin

INSIG: Insulin-induced gene proteins

Jnk: C-Jun N-terminal kinases

LDH: Lactate dehydrogenase

LPS: Lipopolysaccharide

LRR: leucine rich repeats

MAMs: Mitochondrial-associated membranes

MAPK: Mitogen-activated protein kinase

MSU: Monosodium urate

MtDNA: Mitochondrial DNA

mTORC1: Mechanistic target of rapamycin complex 1

MWS: Muckle-wells syndrome

Myd88: Myeloid differentiation primary response 88

NEK7: NIMA-related protein kinase 7

NEM: N-ethylmaleimide

NETs: Neutrophile extracellular traps

NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells

NK: Natural killer

NLR: Nod-like receptor

NLRC: NLR Family CARD Domain Containing

NLRP: Nucleotide-binding oligomerization domain, Leucine rich Repeat and Pyrin domain containing

NOD: Nucleotide oligomerisation domain

NPC: Niemann-Pick protein type C

P2X7: P2X purinoceptor 7

PAMP: Pathogen-associated molecular pattern

PI: Propidium Iodide

PPR: Pattern recognition receptor

PKA: Protein kinase A

PKD: Protein kinase D

PYD: Pyrin domain

RLR: RIG-I-like receptors

RNA: Ribonucleic acid

ROS: Reactive oxygen species

S1P: Site-1-protease

S2P: Site-2 protease

SCAP: Sterol regulatory element-binding protein cleavage-activating protein

TLR: Toll-like receptor

TNF: Tumour necrosis factor

TRIM3: Tripartite Motif Containing 3

VIP: Variable importance in projection

WT: Wild type

Chapter 1
Introduction

Chapter 1 Introduction

1.1 The innate immune system

The immune system has never been more at the forefront of people's minds. With the COVID-19 pandemic, and recent advances in medicine that have changed the healthcare landscape, the complex interplay between the immune system and the world we live in is of vital importance (Koh *et al.*, 2020; Ramamurthy *et al.*, 2021). Typically, the immune system is broken down into two arms: the innate and the adaptive immune system. Innate immunity is an evolutionarily conserved system that serves as the body's first line of defence against pathogens and tissue damage (Carpenter and O'Neill, 2024). Characterised by its rapid and conserved pattern based response, innate immunity serves as the initial defence, recognising a wide array of challenges through a repertoire of specialised receptors (Janeway, 1989; Tabeta *et al.*, 2004; Tall and Yvan-Charvet, 2015; Li and Wu, 2021). Whilst the adaptive immune response is slow to activate, it is extremely potent and is characterised by its immunological memory and the capacity to generate diverse, antigen-specific responses (Sherwood *et al.*, 2022; Lam, Lee and Farber, 2024).

Recent advancements have underscored the pivotal role of innate immunity in various aspects of human health and disease. For example, studies on the microbiome have demonstrated that innate immune pathways shape microbial composition, with implications for conditions such as inflammatory bowel disease and metabolic disorders (Zheng, Liwinski and Elinav, 2020). Additionally, during viral infections, innate immune sensors such as RIG-I have been shown to play essential roles in antiviral defence, contributing significantly to our understanding of host responses to pathogens like SARS-CoV-2 (Lai *et al.*, 2023). Similarly, research in immunometabolism has revealed how innate immune cells, particularly macrophages, regulate metabolic pathways during inflammation, influencing diseases like diabetes and obesity (Chou *et al.*, 2021). These findings firmly establish innate immunity as a cornerstone of the broader immune response (Carpenter and O'Neill, 2024).

With each decade, new groundbreaking discoveries add complexity to the innate immune system such as the complement pathways, cytokines, distinct innate immune cells, and pattern recognition receptors (PRRs), unveiling intricate mechanisms of host defence and regulation that shape our understanding of immunity and disease pathogenesis (Trouw and Daha, 2011; Hivroz *et al.*, 2012; Netea *et al.*, 2020; West and Kemper, 2023; Carpenter and O'Neill, 2024),. Moreover, the distinction between innate and adaptive immunity is increasingly blurring. For example, important findings have underscored the role of the innate immune system in functions that were once considered exclusive to the adaptive immune response,

particularly through the concept of innate immune memory (Netea, Quintin and Van Der Meer, 2011; Sherwood *et al.*, 2022). Although not as long-lasting as adaptive immune memory, innate immune memory is mediated by myeloid cells that can undergo epigenetic changes, enabling extended functionality (Netea *et al.*, 2020). Despite innate immune memory being a relatively new area of study in immunology, it has become clear that what was previously considered a simple cellular response is actually a complicated and interconnected defence system.

1.2 Cell types of the innate immune response

The innate immune system is composed of various specialised cells, each with distinct forms and functions (Marshall *et al.*, 2018). Originating from haematopoietic stem cells, innate immune cells develop from a common precursor called the myeloid progenitor cell (Netea *et al.*, 2020; Ratajczak and Kucia, 2021; Margraf and Perretti, 2022). This progenitor can differentiate into granulocytes—such as neutrophils, eosinophils, mast cells, and basophils—or into phagocytes, which include the monocyte cell line. (Galli, Borregaard and Wynn, 2011). The differentiation of myeloid progenitors relies on a dynamic interaction between intrinsic transcription factors, epigenetic markers, and extrinsic signals, such as cytokines and growth factors (Weiskopf *et al.*, 2016).

1.2.1 Neutrophils

Neutrophils are non-proliferating short lived cells that circulate freely in the body (Summers *et al.*, 2010). Whilst in circulation, they appear as if dormant and only become active upon interaction with endothelial cells at the site of infection or inflammation (Theilgaard-Mönch *et al.*, 2004). Acting as the first line of defence with the potential to phagocytose, degranulate, undergo respiratory burst and produce neutrophil extracellular traps (NETs), neutrophils have a diverse array of “weaponry” at their disposal (Lee, Harrison and Grinstein, 2003; Brinkmann *et al.*, 2004; Malech, Deleo and Quinn, 2014).

As a granulocyte, the neutrophil’s primary function is degranulation, the process whereby preformed granules are released into the extracellular milieu (Malech, Deleo and Quinn, 2014). With four specific categories of granules to release, these occur as bursts of specific signalling molecules that are tailored to a specific need (Lacy, 2006; Eichelberger and Goldman, 2020). Antimicrobial proteins and enzymes are released directly into the extracellular space, which can kill pathogens and further degrade tissues (Lacy, 2006). This process is distinct from the release of reactive oxygen species during a respiratory burst, where neutrophils, and other innate immune cells, produce ROS such as hydrogen peroxide

and superoxide. These molecules are secreted from the cells to kill extracellular pathogens. An exciting recent discovery regarding neutrophils is the formation of NETs. NETs are web-like structures composed of neutrophil DNA and antimicrobial proteins that can trap pathogens and signal other innate immune cells for their destruction (Brinkmann *et al.*, 2004; Vorobjeva and Chernyak, 2020)

Phagocytosis, a primary tool in the arsenal of innate immunity, is a fundamental process in which innate immune cells engulf pathogenic material, forming a membrane-bound compartment known as a phagosome, which in turn can from a phagolysosome (Nordenfelt and Tapper, 2011; Lee *et al.*, 2020). In neutrophils, this formation of a phagolysosome occurs between preformed granules, which contain reactive oxygen species (ROS), digestive enzymes, and a highly acidic environment (Nordenfelt and Tapper, 2011; Gierlikowska, Gierlikowski and Demkow, 2020).

1.2.2 Eosinophils, basophils and mast cells

Three other distinct innate immune cells belong to the granulocyte family. Eosinophils are primarily found in circulation, close to mucosal surfaces, while basophils are present in circulation. Both granulocytes have been extensively studied for their roles in allergic reactions, where the innate immune response is inappropriately stimulated by harmless stimuli, leading to severe consequences (Minai-Fleminger and Levi-Schaffer, 2009). Beyond their erroneous overreactions to allergens, basophils and eosinophils play essential protective roles against parasites (Minkah *et al.*, 2019). Eosinophils directly release harmful reactants to damage parasites, while basophils primarily act as modulators of the immune response, particularly influencing Type 2 T cells (Charles A Janeway *et al.*, 2001). Both cell types contribute to the production of IgE and antibodies specific to parasitic infections (Stone, Prussin and Metcalfe, 2010). Mast cells are distinct from eosinophils and basophils due to their static nature; they function as tissue-resident cells and mediate hypersensitivity reactions (Stone, Prussin and Metcalfe, 2010). While each of these cell types has redundant functions in some areas, their unique abilities to produce specific defences add complexity to the innate immune response.

1.2.3 Natural killer cells

Before moving on to other major classifications of innate immune cells, it is important to highlight two other essential innate immune cells — natural killer cells and epithelial cells. Natural killer (NK) cells are classified as a type of lymphocyte; these innate immune cells

specifically target and kill tumour cells and virus-infected cells (Vivier *et al.*, 2008). NK cells possess the innate ability to recognise missing self signals; they monitor the expression of major histocompatibility complex (MHC) class I receptors, which indicate homeostatic conditions (Johansson *et al.*, 1997). If these receptors are reduced or exhibit abnormal information, NK cells can become activated and induce cell death by directly releasing perforin and granzymes (Voskoboinik, Whisstock and Trapani, 2015). This process leads to pore formation in the target cell and triggers apoptosis. NK cells are unique in the immune system because they serve as a bridge between innate and adaptive immunity (Sun and Lanier, 2009). They originate from lymphoid precursors and can respond rapidly to broadly defined innate signals, with some subsets capable of limited clonal expansion and functional specialisation akin to adaptive lymphocytes.

1.2.4 Epithelial cells

Meanwhile, epithelial cells are the physical barrier of the innate immune response, forming a continuous physical barrier that covers surfaces of the body. This includes skin as well as the mucosal membranes that occur in respiratory, gastrointestinal, and urogenital tracts (Hiemstra, McCray and Bals, 2015; Okumura and Takeda, 2017; Kong, Zhang and Zhang, 2018). Whilst it was previously thought that this stationary cell type merely prevented invasion, it is now becoming apparent that they are highly active, additionally producing a chemical barrier in the from of antimicrobial peptides, such as defensins or lysozymes (Gallo and Hooper, 2012). Epithelial cells also act as initial signalling hubs by responding to infection via the production of pro-inflammatory cytokines and chemokines (Stadnyk, 1994), facilitating rapid interaction by macrophage and neutrophils and triggering adaptive immune responses. Whilst NK cells and epithelial cells do not fall into the classic subtypes of innate immunity, their functions are no less vital.

1.2.5 Monocytes, macrophages, and dendritic cells

Monocytes, macrophages, and dendritic cells represent the prototypical phagocytes. Whilst other innate immune cells have the ability to carry out phagocytosis, phagocytic cells are particularly adapted for phagolysosome formation (Schumann, 2016; Lee *et al.*, 2020). It was initially thought that the sole purpose of monocytes was to serve as precursor cells for differentiated macrophages and dendritic cells (Ginhoux *et al.*, 2015). However, recent research has highlighted that monocytes are not merely transitional cells but also play active roles in immune responses, including antigen presentation and the modulation of inflammation (Cortez-Retamozo *et al.*, 2012; Mitchell, Roediger and Weninger, 2014; Netea *et al.*, 2020;

Villar *et al.*, 2023). This has been further demonstrated by studies showing that monocytes are able to produce cytokines, such as IL-1, IL-6, IL-10, IFN-y and TNF-a in response to activation (Tipton, Pabst and Dabbous, 1990; Prasse *et al.*, 2007; Ginhoux and Jung, 2014; Kraaij *et al.*, 2014; Ginhoux *et al.*, 2015). Whilst more has been elucidated about their own function, monocytes are still most known for their role as precursors to macrophages and dendritic cells (DC).

Monocyte differentiation is mediated by different cytokines that push the monocyte to one of its cellular fates. Initially, it was reported that granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4 induced the differentiation of monocytes to DCs (Romani *et al.*, 1994; Chomarat *et al.*, 2000). However, subsequent research revealed that this process might not fully mimic the development of conventional DCs *in vivo*, as it often generates a distinct subset resembling monocyte-derived DCs rather than the classical DCs (Manh *et al.*, 2013; Goudot *et al.*, 2017). Classical DCs are derived from pre-DCs and are highly phagocytic, primarily known for their role as antigen-presenting cells. (Merad *et al.*, 2013; Wculek *et al.*, 2019). This feature makes them highly capable at inducing the adaptive immune response, through the presentation of pathogenic antigens to adaptive immune cells. DCs express both the MHC as a means to display antigens as well as co-receptors such as the CD80 receptor which is needed for the activation of T cells (Hawiger *et al.*, 2001; Liu *et al.*, 2002; Fujii *et al.*, 2004). Macrophages are distinct from DC within the phagocyte cell line.

Macrophages, which are found in nearly every tissue, are fully-differentiated phagocytic cells with limited capacity for proliferation (Haldar and Murphy, 2014). Upon activation, macrophages play a crucial role in signalling and recruiting other innate immune cells, such as neutrophils, to the site of infection or injury, thereby coordinating the immune response (Prince *et al.*, 2004; Gordy *et al.*, 2011). The outcome of macrophage activation is strongly influenced by the surrounding microenvironment, which drives macrophage polarisation (Lichtnekert *et al.*, 2013). Polarisation refers to the process by which macrophages are induced to express specific genes associated with distinct functional phenotypes. While macrophages were traditionally categorised into M1 (pro-inflammatory) and M2 (anti-inflammatory) subsets (Wager and Wormley, 2014), recent research highlights their functional plasticity and context-dependent activation states, reflecting a broader spectrum of phenotypes that extend beyond this binary model (Murray *et al.*, 2014; Langston, Shibata and Horng, 2017; Sun *et al.*, 2022; Strizova *et al.*, 2023). Additionally macrophages are further divided into either tissue-resident or infiltrating subsets, identifying their distinction can be difficult but possible, due to the tissue specific expression of macrophage markers (Mass *et al.*, 2023). For example, alveolar macrophages express CD169 whereas intestinal

macrophages lack CD169 (Yu *et al.*, 2016), suggesting they are distinct subsets whilst fulfilling the same role. Both in circulation and embedded in tissues, macrophages maintain homeostasis whilst also constantly searching for danger signals.

1.3 Innate signalling

Two key defining features of the innate immune system are that it is a rapid response and that it responds to conserved patterns (Fujii *et al.*, 2004; Gasteiger *et al.*, 2017; Netea *et al.*, 2020). This idea was cemented in the hypothesis that the innate immune response functioned on a system of PRRs. First hypothesised in 1989 by Charles Janeway, these receptors have a defined signal classified as a pathogen-associated molecular pattern (PAMP) (Janeway, 1989). The PRRs operate on a system of recognition in which they are able to detect classes of molecules that are not usually present in 'self' and thus indicative of an infection (Janeway, 1989). This idea was further established by the work of Polly Matzinger who suggested that what the innate immune system actually responded to was danger and established the concept of danger/damage-associated molecular patterns (DAMPs) (Matzinger, 2002). The recognition of PAMPs and DAMPs by PRRs now constitutes a fundamental principle of innate immunity. Although this mechanism may have initially appeared to be a rudimentary form of activation and response, the extensive diversity of PAMPs and DAMPs renders them highly effective targets for innate immune responses (Chuenchor *et al.*, 2014; Li and Wu, 2021). Examples such as lipopolysaccharide (LPS), peptidoglycan, viral DNA, flagellin, adenosine triphosphate (ATP), heat shock proteins, DNA fragments, low density lipoproteins, and uric acid highlight the diversity of ligands that can induce an innate immune response (Hayashi *et al.*, 2001; Desaki *et al.*, 2006; Gust *et al.*, 2007; Fernandes-Alnemri *et al.*, 2009a; Ranjan *et al.*, 2009; Fitrolaki *et al.*, 2016; Braga *et al.*, 2017; Zindel and Kubes, 2020).

PAMPs are highly conserved molecular patterns essential for pathogenicity, making them perfect targets for the induction of an immune response (Janeway, 1989; Sundaram and Kanneganti, 2021). Because PAMPs play essential roles in the life cycle and pathogenicity of microorganisms, they are less likely to be altered or lost through evolutionary adaptation (Mushegian and Medzhitov, 2001). This means that pathogens cannot easily mutate or discard these structures without compromising their own viability. Unlike PAMPs, DAMPs are not associated with pathogenic microorganisms and are instead released in sterile environments via host cells (Matzinger, 2002; Zindel and Kubes, 2020). DAMPs are detected to alert the immune system to tissue injury and initiate processes like tissue repair and inflammation.

1.4 PRRs

PRRs are conserved signalling receptors located both on the membrane and in the endosome of innate immune cells (Brencicova *et al.*, 2013; Amarante-Mendes *et al.*, 2018), that are essential for surveillance of the host microenvironment. Capable of identifying conserved patterns expressed as specific ligand classes, these receptors trigger intricate signalling pathways that activate innate immune cells. This allows not only for innate immune cell activation but also effector functions of the innate immune system and the adaptive immunity via dendritic cell maturation, induction of stimulatory molecules and increase antigen presentation (Ginefra, Lorusso and Vannini, 2020; Li and Wu, 2021). PRRs are essential for tissue development and deletion can lead to non-viable cells, autoimmune disease or uncontrolled infection. (Feerick and McKernan, 2017). The major classes of PRR are Toll-like receptors (TLR), NOD-like receptors (NLR), C-type lectin receptors (CLRs), RIG-I-like receptors (RLRs), AIM2-like receptors (ALRs), and cyclic GMP-AMP synthase.

1.4.1 TLRs

TLRs were the first discovered class of PRRs, making them the most well-known and studied (Medzhitov, Preston-Hurlburt and Janeway, 1997; Qureshi *et al.*, 1999). They are structurally diverse with 10 TLRs in humans and 13 in mice and are located both within the plasma membrane and in endosomes (Chuenchor *et al.*, 2014). The family of receptors is defined by an extracellular leucine rich repeat domain (LRR) and an intracellular cytoplasmic toll-IL-1 receptor/ resistance (IL-1R) (TIR) homology domain (Botos, Segal and Davies, 2011). TLR4 was the first established TLR and has been extensively studied for its response to its ligand lipopolysaccharide (LPS), a key component in Gram-negative bacteria cell walls (Vidya *et al.*, 2018). Despite its early inception, TLR4 is considered to have a complex downstream signalling pathway, as it can interact with multiple adaptor proteins. TLR2 is located on the plasma membrane and can from a heterodimer with TLR1 and TLR6, and can respond to a range of ligands such as lipoproteins or peptidoglycan (Zandieh *et al.*, 2023). Another plasma membrane TLR is TLR5 which recognises bacterial flagellin. The intracellular TLRs are TLR3, TLR7, TLR8 and TLR9, which sense self and non-self nucleic acid ligands. TLR3 recognises dsRNA, TLR7/8 have a high degree of similarity and recognise ssRNA and finally TLR9 recognises ssDNA (e.g. bacterial and viral DNA), specifically the CpG motif (Alexopoulou *et al.*, 2001; Tabeta *et al.*, 2004; Brencicova *et al.*, 2013). This extensive repertoire of ligands makes TLRs the most highly diverse family of PRRs, with a pivotal role in host defence.

1.4.2 CLRs

CLRs are innate immune receptors that primarily play a role in the detection of carbohydrate structures on the surface of fungi, bacteria, viruses and parasites (Brown, Willment and Whitehead, 2018) and are defined by their C-type lectin domains structure which utilises calcium to bind to its ligands (Nagae and Yamaguchi, 2020). Like other PRRs, CLRs exist as transmembrane proteins found on the surface of innate immune cells. However, interestingly they are also secreted as soluble molecules in the circulation (Janeway and Medzhitov, 2002; Kang *et al.*, 2005). As they target carbohydrate structures, they are of particular importance in defence against fungal pathogens which often have β-glucan based cell wall.

1.4.3 RLRs

RIG-I-like receptors function to defend against viral infections and are primarily found within the cytosol (Ramos and Gale, 2011). They detect short dsRNA which are produced in viral infection. Once bound to their ligand, they interact with mitochondrial antiviral signalling protein and induce the production of type-I interferons and IFN- α and IFN-β (Rehwinkel and Gack, 2020). These cytokines can in turn induce further defence and have been implicated in protection from picornavirus, influenza, hepatitis C and rabies (García-Sastre, 2002).

1.4.4 ALR

ALRs are another family of cytosol located PRRs that functions to detect cytosolic dsDNA (Gray *et al.*, 2016). This allows them to act as defences against both cellular damage, in which dsDNA may have escaped the nucleus and during viral infections (Rathinam *et al.*, 2010). The prototypical ALR identified was the absent in melanoma 2 receptor (AIM2), so termed as it was not present in a study which was examining melanoma (DeYoung *et al.*, 1997; Kumari *et al.*, 2020). When the PRR was absent, there was significantly more aggressive tumour growth, highlighting the importance of AIM2 in regulating immune response to tumours. AIM2 is recognized for its ability to assemble an inflammasome, as discussed further in Chapter 1.7.

1.4.5 NLRs

Researchers discovered NLRs in the early 2000s, where they established the NOD1 receptor as a cytosolic PRR capable of detecting peptidoglycan fragments (Aganna *et al.*, 2002; Aksentijevich *et al.*, 2002; Martinon, Burns and Tschopp, 2002a; Agostini *et al.*, 2004). This family of receptors has rapidly expanded to include 22 NLRs identified in humans and 34 NLRs found in mice (Ting *et al.*, 2008). The structure of NLRs is characterised by a tripartite

arrangement, including an invariant central domain—referred to as a NACHT, NOD, or NBS domain—that mediates nucleotide binding and oligomerization (Dekker *et al.*, 2021). Attached to this central domain is a more variable N-terminal domain containing either a caspase activation and recruitment domain (CARD) or a pyrin domain (Yu *et al.*, 2024). N-terminal sites allow for the effector function on the NLR and are required for its activation downstream of interaction with its stimuli, whilst the C-terminus contains an LRR domain, which functions as the specific receptor for NLR agonists. These LRRs have also been implicated in influencing conformational changes and modulating the NLRs response to varied pathogens, highlighting new research into the immune modulation. Furthermore, within LRRs there have been reported auto-inhibitory effects in the absence of NLR agonists (Riedl and Salvesen, 2007). The most studied NLR is the NLRP3, an innate PRR that is responsible for pro-inflammatory signalling and the formation of the inflammasome.

1.5 Formation of the inflammasome

Certain NLRs, and some members of the ALR family, can form structures called inflammasomes. Inflammasomes are multimeric protein complexes consisting of three components. They are formed of their sensing protein, the NLR, ALR or pyrin receptor, responsible for their activation. Additionally, the inflammasome contains an adaptor protein, apoptosis associated speck-like protein containing a caspase-activation and recruitment domain (ASC). ASC acts as a bridge between the sensing protein and the effector protein of the inflammasome, containing both a pyrin domain (PYD) and a CARD domain. This CARD domain allows for binding of another component of the inflammasome, caspase-1. Specific inflammasomes, such as the NLRC4 inflammasome, do not necessarily need ASC and may instead bind to pro-caspase-1 directly via CARD-CARD interaction (Martinon and Tschopp, 2004; Case, 2011). Pro-caspase-1 is synthesised as an inactive enzyme, consisting of a large subunit (p20, N-terminal) and a small subunit (p10, C-terminal) with a bridging linker region (Elliott *et al.*, 2009). Once bound to other inflammasome components, via its CARD domain, pro-caspase-1 undergoes auto-proteolysis. This cleavage results in the release of the p10 and p20 subunits, converting pro-caspase-1 into its active form, caspase-1. Activation of caspase-1 exposes its protease domains, enabling it to cleave and process various substrate proteins (Boucher *et al.*, 2018).

The primary substrates of active caspase-1 include the pro-inflammatory cytokines pro-IL-1β and pro-IL-18, as well as the pore-forming protein gasdermin-D (GSDMD) (Shi *et al.*, 2015; Y. Zhang *et al.*, 2018; Miao *et al.*, 2023). Upon activation, caspase-1 cleaves these cytokine precursors, generating the mature forms IL-1β and IL-18, and producing the N-terminal

fragment of GSDMD. This fragment inserts into the cell membrane, forming pores that facilitate a type of inflammatory cell death known as pyroptosis (He *et al.*, 2015; Sborgi *et al.*, 2016). During pyroptosis, the mature cytokines are released through the GSDMD pores, allowing additional PAMPs and DAMPs to enter the cell. This entry further amplifies inflammasome activation, establishing a positive feedback loop that eventually results in plasma membrane rupture. Contrary to earlier beliefs that membrane rupture was a spontaneous outcome of pyroptosis, it is now recognised to be regulated by the protein ninjurin-1 (NINJ1) (Degen *et al.*, 2023; Fu, Schroder and Wu, 2024). NINJ1 facilitates plasma membrane rupture during pyroptosis, promoting the release of intracellular contents and triggering inflammatory responses.

Pro-IL-1β and pro-IL-18 are synthesised as inactive precursors and require cleavage by caspase-1 to become biologically active before release into the extracellular milieu (Molla *et al.*, 2020). Constitutively expressed in nearly all cell types, IL-18 is produced by the *IL18* gene and binds to the IL-18 receptor with alpha and beta isoforms, which are primarily expressed on immune cells such as T cells, NK cells, and other monocytes (Dinarello *et al.*, 2013). Once bound to the receptor, IL-18 induces the expression of pro-inflammatory genes via the MyD88-dependent pathways, which in turn leads to activation of master immune transcription factor NF-κB. Recent research highlights activator protein-1 as a target of IL-18, regulating genes involved in inflammation and cellular stress. IL-18 is crucial for priming type 1 T helper cells and NK cells, promoting interferon-gamma (IFN-γ) production and cytotoxicity (Dinarello *et al.*, 2013).

In comparison, IL-1β is only produced upon stimulation of NF-κB transcription factor and has a more diverse role, binding to the IL-1 receptor type 1 and its decoy receptor IL-1R2 (Dinarello, 2018). Once bound to its receptor, IL-1β leads to the production of cytokines such as IL-6 and tumour necrosis factor-alpha (TNF-α) (Cahill and Rogers, 2008; Pugazhenthi *et al.*, 2013), and also enhances leukocyte adhesion and promotes migration to sites of injury (Sheikh *et al.*, 2005). Finally, IL-1β also acts to ensure vasodilation for movement of immune cells. Furthermore, it induces the differentiation of T and B cells, the production of prostaglandins and overall temperature changes, underscoring the vital role it plays in regulating the inflammatory response. Whilst IL-1β is a potent pro-inflammatory cytokine, it also has the ability to stimulate wound repair and fibroblast activation, angiogenesis, and extracellular matrix remodelling, leading to post-inflammation recovery (Mia, Boersema and Bank, 2014; Y. Zhang *et al.*, 2019). Because of their key roles in inflammatory diseases, inhibiting pro-inflammatory cytokines, such as IL-1β is an attractive strategy for treatment of a number of inflammatory disease (Dinarello and van der Meer, 2013).

At the same time as cleaving pro-IL-1β and pro-IL-18 to their active forms, caspase-1 also cleaves the pore-forming protein GSDMD. Acting as an executioner protein, GSDMD is split into a regulatory C-terminal domain and the pore-forming N-terminal domain. Upon cleavage, the N-terminal domain is released allowing its translocation to the plasma membrane (Xia *et al.*, 2021). The N-terminal domain of GSDMD has an affinity for lipids in the plasma membrane, specifically phosphoinositides and phosphatidylserine, which facilitates its translocation (Liu *et al.*, 2016). As more GSDMD is cleaved, more N-terminal subunits accumulate at the membrane and begin to oligomerize into large complexes. These complexes insert themselves into the lipid bilayer and form a ring-like structure or pore (Shi *et al.*, 2015). These pores disrupt the membrane barrier and create permeable channels in the cell. Once permeable, there is an influx of ions, water, and small molecules into the macrophage, leading to dysregulation of the osmotic gradient (Kayagaki *et al.*, 2015; Shi *et al.*, 2015). Inflammasomes are defined by the sensing protein that induces their activation.

1.6 NLRP3 inflammasome

1.6.1 Priming of the NLRP3 inflammasome

The NLRP3 inflammasome is distinct among inflammasomes as it requires more than just basal expression for activation, relying instead on a two-step activation process (Mariathasan *et al.*, 2006; Bauernfeind *et al.*, 2009a). This process involves sequential steps known as priming (signal 1) and activation (signal 2). During the priming phase, the expression of NLRP3 and the precursor cytokine pro-IL-1β is upregulated, a process mediated by the transcription factor NF-κB (Liu *et al.*, 2017) (**Fig.1.1**). It is important to note that caspase-1, ASC, and pro-IL-18 are not regulated by NF-κB but are instead constitutively expressed (Bauernfeind *et al.*, 2009). Upon stimulation, NF-κB translocates to the nucleus in a MyD88-, TRIF- and TRAM-dependent pathway, and activates genes associated with the inflammasome, such as *NLRP3* and *IL1B*. In addition to its role in the induction of inflammasome components, studies have shown that NF-κB is involved in the removal of damaged mitochondria, which serve as activation signals for the inflammasome, thereby preventing aberrant inflammasome activation (Zhong *et al.*, 2016). Furthermore, apoptotic regulators like caspase-8 and FADD are involved in the regulation of NLRP3 priming via activation of NF-κB (Gurung *et al.*, 2014).

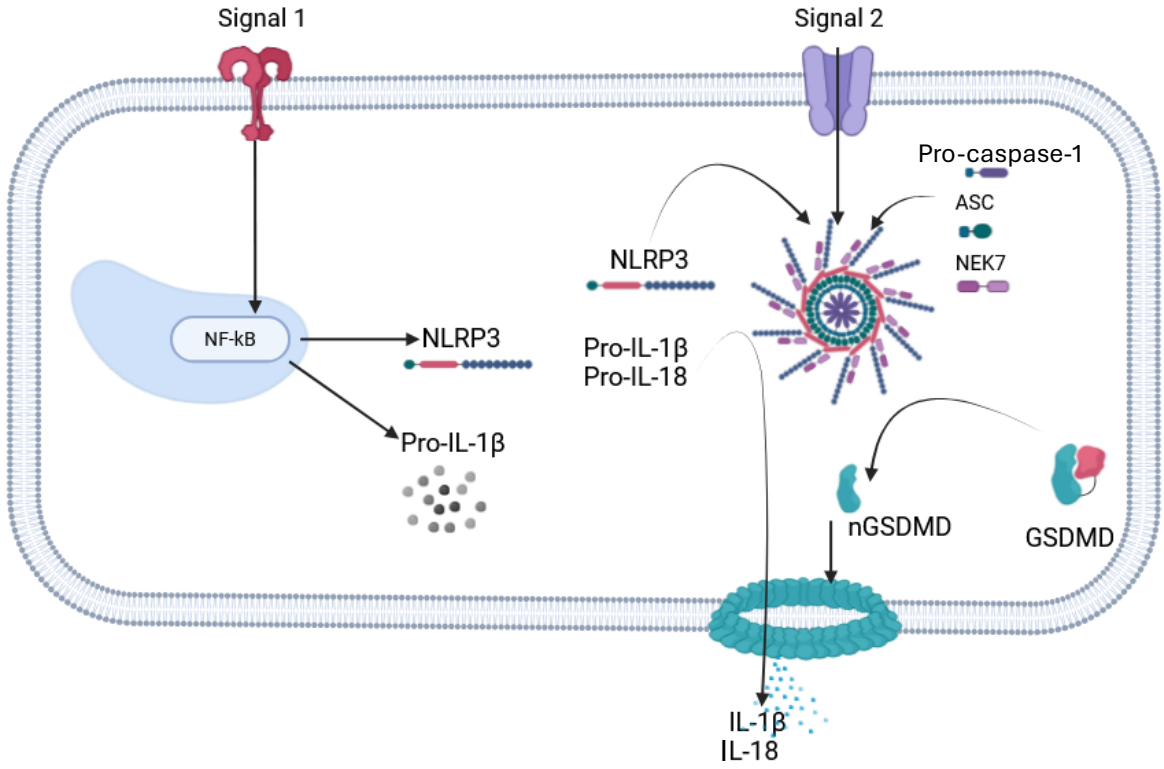


Figure 1.1 Activation of the NLRP3 inflammasome.

For NLRP3 inflammasome activation to occur, there must first be a priming event, also known as signal 1. This signal is mediated by pattern recognition receptors and leads to the activation of the master transcription factor NF-κB. Once activated, there is production of the NLRP3 sensor protein and precursor cytokine pro-IL-1β. Cells can maintain this primed state without undergoing activation for some time before a secondary activation or signal 2 is received, upon which oligomerisation of the inflammasome components to the fully-formed inflammasome structure occurs. This inflammasome auto-cleaves caspase-1 which in turn cleaves precursor cytokines pro-IL-1β and pro-Il-18 into their active forms, as well as the pore forming protein GSDMD into its active N-terminal fragment. N-terminal GSDMD produces pores in the plasma membrane and allow the efflux of cytokines and influx of PAMPs and DAMPs, leading to inflammatory cell death pyroptosis. Created with BioRender.com.

1.6.2 Activation of the NLRP3 inflammasome

Under basal conditions, NLRP3 expression levels remain low, which is insufficient for initiating inflammasome assembly, even when ASC and pro-caspase-1 are adequately present. Interestingly, overexpression of NLRP3 can facilitate inflammasome activation in the presence of a secondary signal alone, irrespective of the priming signal (Bauernfeind *et al.*, 2009). Furthermore, it has been shown that monocytes, but not monocyte-derived macrophages, can undergo NLRP3 activation without a priming signal (Gritsenko *et al.*, 2020). In this context, monocytes release mature IL-18 in a GSDMD-dependent manner, despite the absence of pro-IL-1β (Gritsenko *et al.*, 2020). Once NLRP3 is synthesised, a secondary activation signal is required for the assembly of the inflammasome complex in macrophages.

Initially, NLRP3 resides in the cytoplasm and awaits activation signals. Notably, in addition to its unique two step licensing mechanism, NLRP3 is characterised by its ability to respond to a diverse array of stimuli compared to other PRRs (Kelley *et al.*, 2019). Research has demonstrated that various factors can induce NLRP3 activation, including lysosomal damage, ion fluxes, and mitochondrial ROS (Hornung *et al.*, 2008; T. Murakami *et al.*, 2012; Karasawa *et al.*, 2018a; Y. Yang *et al.*, 2019). This broad range of potential activation signals has led to speculation that there may be a shared upstream molecule that facilitates NLRP3 activation; however, no such molecule has been definitively identified.

Ion fluxes

Ion fluxes have long been linked to inflammasome activation, with potassium (K^+) efflux—induced by factors such as ATP, nigericin, and particulate or crystalline molecules—recognised as a common trigger for NLRP3 activation (Walev *et al.*, 1995; Colomar *et al.*, 2003; Muñoz-Planillo *et al.*, 2013). K+ efflux was first linked to NLRP3 through experiments showing that K+ efflux is essential for multiple triggers of the inflammasome to function. This was further validated by demonstrating that low levels of extracellular K+ was able to induce inflammasome activation (Muñoz-Planillo *et al.*, 2013c). The precise mechanism by which K^+ influences NLRP3 activation remains unclear; however, recent studies indicate that NIMA-related protein kinase 7 (NEK7) may be involved downstream of K^+ efflux (He *et al.*, 2016). Notably, K^+ efflux specifically activates the NLRP3 inflammasome without impacting the activity of other inflammasomes, such as AIM2 or NLRC4 (Xu *et al.*, 2020).

NEK7 has been implicated to be essential for NLRP3 inflammasome activation by several groups (He *et al.*, 2016; He, Hara and Núñez, 2016; Schmid-Burgk *et al.*, 2016). NEK7 directly interacts with NLRP3, serving as a structural scaffold that promotes the oligomerization of NLRP3 subunits, a critical step in the assembly of the active inflammasome complex. In the

absence of NEK7, the formation of an active inflammasome is significantly impaired, even when sufficient levels of NLRP3, ASC, and a secondary activation signal are present (Shi *et al.*). Additionally, NEK7 has not been reported to interact with other inflammasome complexes, further supporting the notion that K^+ efflux specifically regulates NLRP3 activation (He *et al.*, 2016).

Other ion fluxes such as calcium (Ca^{2+}), chloride (Cl^-) and sodium (Na^+) have also been implicated in activation of the NLRP3 inflammasome (Verhoef *et al.*, 2005; Schorn *et al.*, 2011; Tomohiko Murakami *et al.*, 2012). Cytoplasmic Ca^{2+} has been shown to promote IL-1β secretion in ATP-activated macrophages and UV-exposed keratinocytes. Calcium-sensing receptors and store-operated Ca^{2+} entry channels have been implicated in this process, potentially through mitochondrial damage (Lee *et al.*, 2012). However, some studies suggest that extracellular Ca^{2+} may activate NLRP3 indirectly via K^+ efflux, and that Ca^{2+} mobilisation may not be necessary for all NLRP3 activators (Muñoz-Planillo *et al.*, 2013a). Therefore, while Ca^{2+} can influence NLRP3 activation, its role is less central than K^+ efflux.

Alternatively, Cl^- has been demonstrated to regulate NLRP3-dependent ASC oligomerization and inflammasome priming (Green *et al.*, 2018). The relationship between Cl^- and NLRP3 was found to be K^+ independent, acting to induce NLRP3-ASC transient oligomerisation until K^+ efflux induced full inflammasome activation (Green *et al.*, 2018). Further, inflammasome activation relies on NEK7 to detect K^+ efflux (He *et al.*, 2016). The role of Cl^- in NLRP3 activation was further supported by studies using fenamates, non-steroidal anti-inflammatory drugs that specifically target Cl^- channels, which prevent K^+ efflux and subsequent NLRP3 activation (Daniels *et al.*, 2016; Swanton *et al.*, 2020). These findings built upon the identification of chloride intracellular channel proteins CLIC1 and CLIC4, which translocate to the nucleus and cellular membranes in response to NLRP3 priming and activation via LPS and ATP. This translocation facilitates Cl^- efflux, further promoting NLRP3 activation (Domingo-Fernández *et al.*, 2017).

Mitochondrial damage

Mitochondrial damage and subsequent ROS production have been implicated in the activation of the NLRP3 inflammasome in response to alterations in cellular homeostasis and interactions with ligands such as ATP (Zhou *et al.*, 2011; Abais *et al.*, 2015; Anand, 2020). ROS can interact with NLRP3 both directly and indirectly, promoting conformational changes that facilitate its assembly into the active inflammasome complex. This process may occur through alterations in the redox environment and the induction of protein modifications, such

as thiol oxidation, which activate NLRP3 (Abais *et al.*, 2015). Additionally, TLR signalling can stimulate the synthesis of mitochondrial DNA (mtDNA) through cytidine/uridine monophosphate kinase 2 (Baik *et al.*, 2023). Oxidised mtDNA fragments have been shown to bind to NLRP3 in the cytosol following exposure to NLRP3 activation signals (Coll, Holley and Schroder, 2018; Xian *et al.*, 2022)

Cardiolipin, a phospholipid present in the mitochondrial inner membrane, has been implicated in NLRP3 activation (S S. Iyer *et al.*, 2013; Zhong *et al.*, 2018a). During NLRP3 activation, cardiolipin relocates from the inner membrane to the outer mitochondrial membrane, where it can directly bind NLRP3 via the LRR domain, facilitating its activation (Zhong *et al.*, 2018a). Blocking cardiolipin synthesis has been shown to reduce inflammasome activation (S S. Iyer *et al.*, 2013). Interestingly, caspase-1 has also been observed to bind cardiolipin, suggesting that this phospholipid may play a role in promoting the oligomerization of NLRP3 components (Elliott *et al.*, 2018; Hamilton and Anand, 2019). Further studies have expanded on the role of cardiolipin in NLRP3 activation, identifying that cardiolipin movement depends on an initial priming signal (Elliott *et al.*, 2018)

Lysosomal damage

Lysosomal damage, often induced by particulate matter such as silica, alum, and cholesterol crystals, has been implicated in the activation of the NLRP3 inflammasome (Hornung *et al.*, 2008; Maxfield, Barbosa-Lorenzi and Singh, 2019). When lysosomes are damaged, their contents, including cathepsins—lysosomal cysteine proteases—are released into the cytosol. Early studies suggested that inhibiting cathepsin B alone could prevent NLRP3 activation; however, it is now understood that inhibition of multiple cathepsins is necessary, indicating a level of redundancy in their role in NLRP3 activation (Chevriaux *et al.*, 2020). This has led to questions about whether lysosomal damage is an independent pathway for inflammasome activation or simply an upstream event leading to ROS production or ion efflux (Muñoz-Planillo *et al.*, 2013).

The diverse mechanisms that can activate NLRP3 highlight the complexity of this inflammasome. It has been proposed that K^+ efflux might serve as a common upstream event for NLRP3 activation, but this hypothesis remains under investigation. The variety of signals capable of activating NLRP3 suggests it is a highly adaptable sensor, with the potential to act as a master regulator in inflammasome biology.

1.6.3 The non-canonical inflammasome

Another pathway for NLRP3 inflammasome activation, known as the non-canonical inflammasome, has also been identified. The canonical pathway, as previously outlined in this thesis, involves a two-step process comprising priming and activation signals. In contrast, the non-canonical pathway requires only an initial stimulation by cytosolic or transfected LPS to trigger activation (Kayagaki *et al.*, 2011; Downs *et al.*, 2020) (**Fig.1.2).** In this pathway, murine caspase-11 (and caspase-4 and caspase-5 in humans) directly binds LPS via its CARD domain, recognizing the lipid A tail moiety of LPS (Shi *et al.*, 2014; Downs *et al.*, 2020). Initially observed in mouse macrophages, the deletion of caspase-11 was found to confer protection against septic shock and significantly reduce inflammasome activation in response to *Escherichia coli*, *Citrobacter rodentium*, *Salmonella typhimurium*, *Legionella pneumophila* or *Vibrio cholerae* (Broz, Von Moltke, *et al.*, 2010; Gurung *et al.*, 2012; Rathinam *et al.*, 2012; Aachoui *et al.*, 2013; Case *et al.*, 2013; Kayagaki *et al.*, 2015). Interestingly, it was identified that endosomal LPS was the primary trigger of non-canonical pathways. Whilst LPS is a well known PAMP of Gram-negative bacteria, these pathogens are not typically intracellular where caspase-11 is functional. It has been suggested that upon internalisation by cells, LPS contained in outer membrane vesicles from Gram-negative pathogens can access the cytosol, thereby interacting with caspase-11 in to activate the non-canonical inflammasome (Vanaja *et al.*, 2016).

It was previously believed that caspase-4 and caspase-5 played redundant roles in the activation of the non-canonical inflammasome. However, recent research indicates that they may have distinct functions in response to specific stimuli, such as markers of organ damage in ACLF or sepsis, both of which are associated with immunosuppression (Ghait *et al.*, 2023). Unlike the canonical inflammasome pathway—where ligand recognition, complex assembly, and effector functions are conducted by the separate NLRP3, ASC and Caspase-1 proteins—the non-canonical pathway involves these processes within a single protein.

Caspase-11, in particular, binds to LPS via its CARD domain, undergoing autocleavage, and subsequently activating downstream targets without the assembly of a full inflammasome complex (Kayagaki *et al.*, 2011; Shi *et al.*, 2014). Recent studies have suggested that intermediate sensing proteins, such as guanylate-binding proteins (GBPs), may help to initiate non-canonical inflammasome activation, although this mechanism is not yet fully understood (Martin and Parton, 2006; Santos *et al.*, 2020; Wandel *et al.*, 2020). GBP-deficient macrophages have diminished caspase-11 activation, being studies suggesting that GBPs

are needed for the lysis of vacuoles to allow LPS release (Pilla *et al.*, 2014; Vanaja *et al.*, 2016).

It was previously thought that only LPS could trigger oligomerisation of the non-canonical inflammasome, but recent research has suggested a more nuanced system for regulation. For example, oxPAPC has been reported to induce caspase-11-dependent release of IL-1β in dendritic cells, without full pyroptosis occurring whereas the same phospholipid confers protection from pyroptosis in macrophages (Zanoni *et al.*, 2016; Chu *et al.*, 2018). Furthermore, the glycolipid lipophosphoglycan (LPG), which is present on the surface of Leishmania parasites, can lead to activation of caspase-11. However, this only occurred *in vitro* and not *in vivo*, suggesting the existence of additional molecules that aid in protection from the parasite (de Carvalho *et al.*, 2019). It recently has become more apparent that both the canonical and non-canonical inflammasome are regulated by not only their stimulus but also a host of other factors, such as their subcellular localisation and post-translation modifications (PTMs).

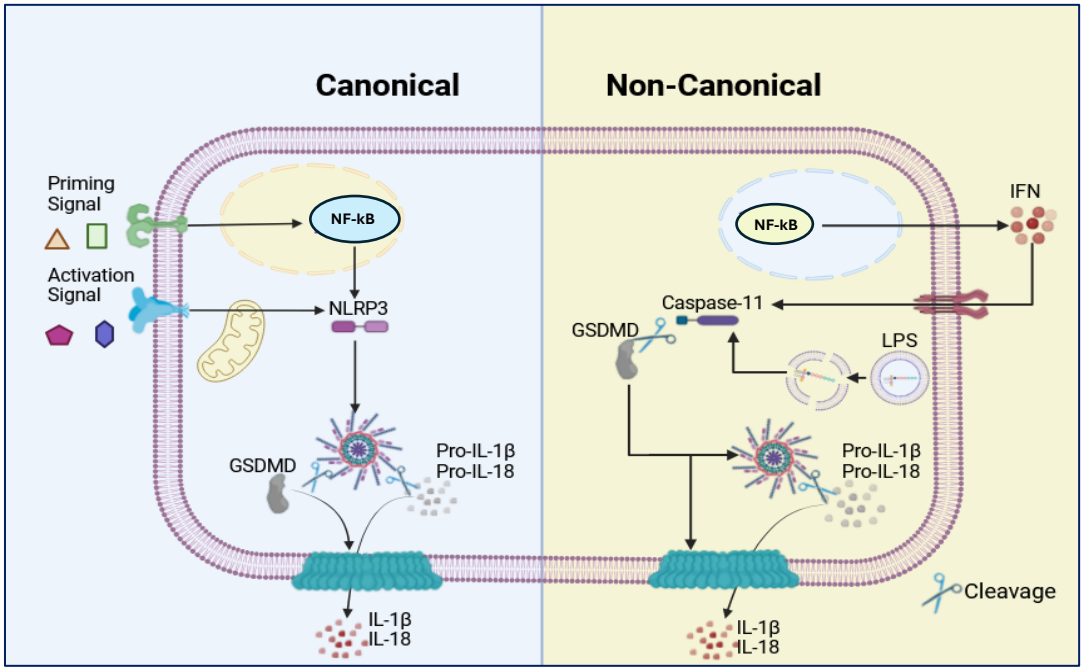


Figure 1.2: The canonical and non-canonical NLRP3 inflammasome

The canonical NLRP3 pathway requires both priming and activation signals to induce pyroptosis and the production of pro-inflammatory cytokines. The non-canonical inflammasome leads to a similar outcome, but it can be activated either via IFN signalling or through the escape of cytosolic LPS. Once either of these signals occurs, caspase-11 in mice or caspase-4/5 in humans cleaves GSDMD. This not only allows the formation of pores in the canonical inflammasome but also permits the influx of PAMPs and DAMPs, which subsequently lead to the formation of the canonical NLRP3 inflammasome and the cleavage of precursor cytokines pro-IL-1β and pro-IL-18. Created with BioRender.com.

1.6.4 Organelles associated with NLRP3 activation

Distinct organelles have been shown to play critical roles in the activation of the NLRP3 inflammasome, contributing to its assembly and signalling. These interactions highlight how cellular stress signals originating from organelles, such as mitochondria, lysosomes, and the ER converge to regulate inflammasome activation (Kwon *et al.*, 2009; Zhou *et al.*, 2011; Akbal *et al.*, 2022).

Mitochondria-associated ER membranes

The process of overall inflammasome assembly is well established, with defined priming and activation stages. New research has revealed that NLRP3 assembly does not occur randomly in the cytosol and that NLRP3 instead translocates upon activation to specific organelles to aid in its ability to undergo oligomerisation (Chen and Chen, 2018b; Guo *et al.*, 2018; Leishman *et al.*, 2024; Zhang *et al.*, 2024). It was initially thought that NLRP3 translocated to free mitochondria—those not associated with other organelles—but it has since been suggested that NLRP3 instead translocates to mitochondria-associated ER membranes (MAMs), a process essential for sensing mitochondrial damage and interacting with mtDNA (Y. Zhou *et al.*, 2020). This concept was further expanded during investigations into cholesterol biosynthesis, which revealed a link between cholesterol metabolism and the *trans*-Golgi network (TGN) (Chen and Chen, 2018; de la Roche *et al.*, 2018; Guo *et al.*, 2018).

Trans Golgi network and endosomes

The Golgi is made up of a series of fused and flattened membranes and consists of a *cis*-Golgi network and TGN (Wang and Huang, 2017). Typically, proteins transition from the *cis*-Golgi network to the TGN and undergo packaging into vesicles. Recent research has shown that the TGN is needed for the spatial regulation of NLRP3 activation. In particular, has been demonstrated that NLRP3 binds to negatively charged phosphatidylinositol 4-phosphate (PI4P) on the TGN and that this binding allows for activation by facilitating oligomerization and inflammasome assembly (Chen and Chen, 2018). It is now hypothesised that, upon activation, NLRP3 transiently associates with MAMs before transitioning to the Golgi over time, where it forms the complete inflammasome complex (Akbal *et al.*, 2022). This hypothesis has been further supported by evidence showing that inhibition of NLRP3 translocation, prevents inflammasome assembly (Zhang *et al.*, 2017a; Chen and Chen, 2018b; Bittner *et al.*, 2021). Furthermore, utilising a NLRP3 tagged with OSBP (Golgi binding protein) can facilitate inflammasome activation.(Zhang. *et al.*, 2023)

Recent structural analyses have provided insights into the translocation of NLRP3 to the Golgi. It is proposed that NLRP3 exists as either a monomer or dimer in the cytosol, transitioning to an inactive double-ring cage oligomer upon activation (Andreeva *et al.*, 2021; Hochheiser *et al.*, 2022). While the positioning of NLRP3 is debated between MAMs and the dTGN, it is well established that complete NLRP3 inflammasome assembly occurs at microtubule-organising centres (MTOC) (Magupalli *et al.*, 2020). This has led to a compelling hypothesis suggesting that NLRP3 is initially recruited to the Golgi membrane to form a double-ring cage structure, which is then translocated to the MTOC for reassembly mediated by PYD interactions (Que *et al.*, 2024).

Studies have demonstrated that NLRP3 activators promote the accumulation of phosphatidylinositol 4-phosphate (PI4P) on the dTGN, and that NLRP3 binds to PI4P via its polybasic region (Godi *et al.*, 1999; Chen and Chen, 2018a). This binding provides a platform for NLRP3 oligomerization with ASC. However, this finding has prompted some questions, as the study utilised antibodies to TGN38 (TGN46 in humans), a marker that undergoes endosomal-TGN transport (Reaves and Banting, 1994; Chen and Chen, 2018a). Upon exposure to NLRP3-stimulating factors, TGN38/46 accumulates in early endosomes prompting discussion whether NLRP3 is found in the dTGN or endosomes that have accumulated TGN38 (Lee *et al.*, 2023). Interestingly, although disruption of the TGN-to-endosome pathway or endosomal membranes can activate NLRP3 in cells overexpressing the protein, cells expressing endogenous NLRP3 require a priming signal. This suggests that NLRP3 undergoes modification to facilitate its translocation to endosomes and potentially its interaction with PI4P (Zhang. *et al.*, 2023).

MTOC

The mechanism by which NLRP3 exits the Golgi remains unclear; however, its migration to the MTOC and subsequent assembly onto the NLRP3/ASC speck has long been suspected (Magupalli *et al.*, 2020). Upon activation NLRP3 is thought to be transported to the MTOC, where it interacts with NEK7. Additional studies have identified factors that regulate this process, including the interaction between NLRP3 and the microtubule affinity-regulating kinase 4 (MARK4), which facilitates NLRP3 transport to the MTOC (Li *et al.*, 2017). The transport system of HDAC6-dynein is also crucial for NLRP3 localisation and activation, functioning independently of HDAC6's deacetylase activity but reliant on its ubiquitin-binding domain (Magupalli *et al.*, 2020). Moreover, mitochondrial dysfunction can lead to reduced NAD+ levels, inactivating sirtuin 2 and promoting dynein-mediated NLRP3 transport (Misawa *et al.*, 2013). However the relationship between NLRP3 and MTOC has recently come under

scrutiny, with work suggesting that upon activation of NlRP3 there is destabilisation of the centrosome, as demonstrated by loss of centrosomal scaffold protein pericentrin (Bai *et al.*, 2024). This centrosomal destabilising suggests that the relationship between NLRP3 and MTOC is more complex then previously suggested and opens up new questions as to how MTOCs influences inflammasome activation. Recent research has also uncovered various post translational modifications (PTMs) that regulate NLRP3 interactions with NEK7 and other components of the inflammasome.

1.6.5 Post translational modification of the NLRP3 inflammasome

PTMs are crucial for tailoring a protein's function, allowing for a higher level of editing and regulation after it has been produced (Levental *et al.*, 2010; Barry *et al.*, 2018; Sun and Zhang, 2022; J. M. Lee *et al.*, 2023). As the name suggests, PTMs involve the addition or alteration of a protein after it has been translated. Unlike genetic mutations or changes in the primary structure of a protein, PTMs involve the addition of various functional groups or the modification of existing ones, leading to significant changes in the protein's properties, activity, stability, and localisation (J. M. Lee *et al.*, 2023; Xia *et al.*, 2023). The relationship between NLRP3 and PTMs has been studied to explore their potential role in regulating the inflammasome beyond its priming and activation signals

Ubiquitination

NLRP3 ubiquitination is critical for regulating its activity, with distinct types of ubiquitin chains influencing its function. Ubiquitination is the process in which the protein ubiquitin is attached to the target protein, often signalling a protein for degradation (Ciehanover, Hod and Hershko, 1978; Hershko *et al.*, 1983). E3 ubiquitin ligases are the main enzymes orchestrating NLRP3 inflammasome activity, with Lys48-, Lys63-, and Lys27-linked ubiquitination playing significant roles (Humphries *et al.*, 2018; Z. Zhou *et al.*, 2020; Xu *et al.*, 2022). Lys48-linked chains typically signal degradation via the proteasome, inhibiting NLRP3 activation. In contrast, Lys63-linked ubiquitination supports NLRP3 activation, with E3 ligases like Pellino-2 and Ubc13 facilitating this process (Humphries *et al.*, 2018). Recent studies highlight the role of K27-linked ubiquitination, which can either inhibit or promote NLRP3 activity depending on the E3 ligase involved (Song *et al.*, 2016; Zhou and Zhang, 2022). As NLRP3 plays such a fundamental role in host defence, regulating its expression is essential for maintaining immune homeostasis and minimising detrimental effects. Ubiquitination allows for precise control of its expression, ensuring that any aberrant levels of NLRP3 are quickly degraded (Song *et al.*, 2016). Overall, TLR stimulation appears to induce both deubiquitination and ubiquitination of NLRP3 at various stages of its activation. Recent findings highlight the roles of ubiquitin-

specific-processing proteases (USP) 7 and USP47 in regulating NLRP3 ubiquitination in response to activating signals. These findings indicate that both priming and activation stages can influence NLRP3's PTMs (Palazón-Riquelme *et al.*, 2018).

Phosphorylation

Phosphorylation is the addition of phosphate groups to serine, threonine, or tyrosine residues (Fischer, E, H. Krebs, 1955; E.G. Krebs, Kent, 1958). Phosphorylation of NLRP3 at specific sites, including Ser725, Ser194, and various tyrosines within the PYD-NACHT linker, is essential for its activation (Song *et al.*, 2017; Stutz *et al.*, 2017; Zhu *et al.*, 2021) Misshapen-like kinase 1 (MINK1), a member of the Germinal Centre Kinase family, phosphorylates NLRP3 at Ser725, which is crucial for the priming step of NLRP3 activation (Zhu *et al.*, 2021) with ROS production enhancing MINK1 activity, promoting this phosphorylation event. C-Jun N-terminal kinase 1 (JNK1) phosphorylates Ser194, facilitating NLRP3 deubiquitination necessary for activation (Song *et al.*, 2017). Bruton tyrosine kinase (BTK) phosphorylates several tyrosines in the PYD-NACHT linker region, leading to NLRP3 translocation and inflammasome assembly. However, its regulatory effect depends on the LPS signalling (Ito *et al.*, 2015). Conversely, AKT-mediated phosphorylation at Ser5 inhibits NLRP3 oligomerization and ASC recruitment, thereby restricting inflammasome activation (Zhao *et al.*, 2020). Various kinases, including protein kinase D (PKD) and protein kinase A (PKA), phosphorylate murine Ser293 (Ser295 in humans), with PKD promoting inflammasome assembly and PKA suppressing activity through dephosphorylation and ubiquitination (Mortimer *et al.*, 2016; Zhang *et al.*, 2017a). In contrast, dephosphorylation of Ser5, Ser803, and Tyr861 enhances NLRP3 activation. In both cases, phosphatases such as PP2A and PTPN22 play central roles in these processes, acting to facilitate the dephosphorylation of NLRP3 residues (Song and Li, 2018). Phosphorylation and dephosphorylation also influence conformational changes in NLRP3, particularly through interactions with NEK7 and the structural dynamics of the double-ring cage this underscores the complexity of NLRP3 regulation (Niu *et al.*, 2021).

Acetylation

Acetylation, the attachment of acetyl groups or acetylation, of NLRP3 plays a key role in enhancing NLRP3 inflammasome activation (Vidali, Gershey and Allfrey, 1968). Lysine acetyltransferase 5 (KAT5) acetylates NLRP3 at Lys24, promoting its interaction with NEK7 and facilitating oligomerization (Yening Zhang *et al.*, 2023). Inhibiting KAT5 with NU9056 reduces NLRP3-dependent caspase-1 maturation and IL-1β release both *in vivo* and *in vitro*. In contrast, the NAD+-dependent deacetylase Sirtuin 2 (SIRT2) counteracts this process by deacetylating NLRP3 in bone marrow-derived macrophages (BMDMs) (Dempsey, 2020).

Deleting SIRT2 or inhibiting it with AGK2 increases IL-1β production and caspase-1 cleavage, although pro-IL-1β expression remains unaffected (Dempsey, 2020). Notably, Sirt2 knockout does not alter caspase-1 maturation or IL-1β secretion upon stimulation with NLRC4 or AIM2 inducers. In mice lacking SIRT2, NLRP3 inflammasome activation is enhanced, body fat accumulates, and plasma glucose and insulin levels rise, especially in those fed a high-fat diet. Furthermore, mutating acetylation sites on NLRP3 (K21/22/24) to arginine mimics a deacetylated state. Aged mice with this mutation exhibit improved glucose clearance compared to those with wild-type NLRP3, suggesting that SIRT2-mediated deacetylation of NLRP3 may alleviate aging-related inflammation and insulin resistance (He *et al.*, 2020).

Alkylation

Alkylation, the attachment of an alkyl group, is used to study various post-translational modifications (PTMs), has been shown to regulate the NLRP3 inflammasome. Alkylation negatively impacts inflammasome activation by reducing ATP-binding affinity, primarily targeting the NACHT domain. (Duncan *et al.*, 2007). This domain uses an ADP/ATP switch mechanism for activation, where ADP maintains inactivity and ATP binding activates NLRP3 through hydrolysis (Dekker *et al.*, 2021). Compounds like MCC950 inhibit NLRP3 by blocking ATP hydrolysis, while alkylating agents like BOT-4-one enhance NLRP3 ubiquitination, reducing activation. (Shim *et al.*, 2017; Coll *et al.*, 2019). The metabolite itaconate and its derivative, 4-OI, also inhibit NLRP3 by alkylating cysteine residues, preventing NEK7 interaction and IL-1β secretion (Mills *et al.*, 2018; Hooftman *et al.*, 2020).

SUMOylation

The small ubiquitin-like modifier (SUMO) protein, with four variants in humans (SUMO1-4), regulates protein function through SUMOylation, a reversible PTM involving attachment to lysine residues.(Meluh and Koshland, 1995; Hay, 2013; Barry *et al.*, 2018). SUMOylation of NLRP3 has dual roles: modifications at Lys204 enhance activation, while modifications at Lys689 inhibit it. Ubc9 mediates SUMOylation at Lys204, and mitochondrial-anchored protein ligase facilitates SUMOylation at Lys689. Surprisingly, Lys689 SUMOylation can promote caspase-1 maturation and IL-1β release (Barry *et al.*, 2018; Shao *et al.*, 2020; Ortega *et al.*, 2023). DeSUMOylation by SENP6 and SENP7 enhances NLRP3 activation (Shao *et al.*, 2020). Additionally, TRIM28 supports inflammasome activation by preventing NLRP3 degradation through SUMOylation (Qin *et al.*, 2021). This intricate regulation fine-tunes inflammasome responses.

S-Nitrosylation

S-nitrosylation of NLRP3 inhibits inflammasome activity by covalently binding a nitric oxide (NO) group to cysteine thiols, forming *S*-nitrosothiols (Martínez-Ruiz and Lamas, 2004). The NO donor *S*-nitroso-N-acetylpenicillamine (SNAP) has been shown to suppress nigericin-induced caspase-1 maturation and the release of pro-inflammatory cytokines IL-1β and IL-18 in macrophages primed with the TLR1/2 agonist PAM3CSK4. Notably, this priming step facilitates the *S*-nitrosylation of NLRP3, particularly at its C-terminal region (Hernandez-Cuellar *et al.*, 2012). Further studies in HEK293T cells demonstrate that SNAP inhibits IL-1β processing only in cells expressing NLRP3, confirming that *S*-nitrosylation of NLRP3 is essential for regulating inflammasome activity (Mishra *et al.*, 2013). Despite these findings, the precise mechanisms by which *S*-nitrosylation modulates NLRP3 remain unclear, necessitating further research into its structural and functional impacts.

S-Glutathionylation

Protein *S*-glutathionylation is a reversible oxidative modification that forms mixed disulfides between glutathione and cysteine residues (Zhang and Forman, 2012, Ullevig *et al.*, 2016). Glutathione transferase Omega 1 (GSTO1-1) plays a key role in inflammation, with its deletion reducing proinflammatory cytokines and LPS-induced inflammation in mice (Ullevig *et al.*, 2016). GSTO1-1 promotes deglutathionylation of NEK7, enhancing NLRP3 inflammasome activation. Superoxide dismutase 1 (SOD1) activates caspase-1 by inhibiting its glutathionylation, while in SOD1-deficient cells, increased ROS leads to caspase-1 glutathionylation and inhibition (Meissner, Molawi and Zychlinsky, 2008). Curcumin reduces NLRP3 *S*-glutathionylation and increases caspase-1 *S*-glutathionylation, decreasing IL-1β production and improving survival in LPS-induced shock models (Gong *et al.*, 2015).

1.6.6 Clinical relevance of the NLRP3 inflammasome

Inflammatory and autoimmune disease

Dysregulation of NLRP3 activity has been extensively investigated and is linked to a variety of diseases and disorders (Heneka *et al.*, 2012; Broz and Dixit, 2016; Rampanelli *et al.*, 2018). These diseases encompass a wide range of conditions, all characterised by dysregulated or excessive immune responses that lead to tissue damage (Bortolotti, Faure and Kipnis, 2018). Inflammatory diseases are often chronic and lifelong, such as rheumatoid arthritis, inflammatory bowel disease, and lupus erythematosus. They are typically influenced by genetic, environmental, and immunological factors (McLean and El-Omar, 2009). Additionally, inflammatory conditions can develop as immunosenescence and a part of the ageing progress

(Li *et al.*, 2023). Many diseases associated with inflammation are linked to the transition from acute to chronic responses, including type-2 diabetes, chronic kidney disease, neurodegenerative diseases, asthma, chronic obstructive pulmonary disease, and inflammatory bowel disease (Abudukelimu *et al.*, 2018). Given the varied nature of inflammation and its involvement in so many conditions, it remains a crucial area of study, with significant research already dedicated to understanding its mechanisms. Therapeutics targeting inflammation induced by the inflammasome are currently in development. One such treatment is anakinra, an IL-1β receptor antagonist, which has shown efficacy in treating rheumatoid arthritis and can block IL-1β activity downstream of the NLRP3 inflammasome (Kary and Burmester, 2003; Ramírez and Cañete, 2018). That said, treatments that directly target the NLRP3 inflammasome are limited, with MCC950 standing out as a potentially promising therapeutic option (Coll *et al.*, 2019).

Metabolic disturbances often lead to the overproduction or accumulation of proteins that prime and activate the NLRP3 inflammasome. For example, the accumulation of fatty acids in obesity can activate toll-like receptors (TLRs), resulting in the priming of the inflammasome. In obese mice, deletion of NLRP3 has been shown to reduce obesity-related inflammation and improve insulin sensitivity, underscoring the important role of NLRP3 in these processes (Shi *et al.*, 2006). Moreover, altered metabolism can generate crystals and protein aggregates, which drive inflammation in conditions such as atherosclerosis, gout, and Alzheimer's disease. Cholesterol crystals formed in atherosclerotic plaques are recognised as danger signals, triggering NLRP3 inflammasome activation via lysosomal disruption and cathepsin release (Duewell *et al.*, 2010a; Rajamäki *et al.*, 2010). Similarly, monosodium urate (MSU) crystals are a key inflammatory factor in gout, activating the NLRP3 inflammasome and leading to joint inflammation and pain through IL-1β and prostaglandin production which in turn activate nociceptors, or pain receptors (Ren and Torres, 2008; Schorn *et al.*, 2011). In type 2 diabetes, metabolic stressors such as high glucose levels and saturated fatty acids similarly activate the NLRP3 inflammasome in adipose tissue and pancreatic cells, contributing to insulin resistance and chronic low-grade inflammation (Vandanmagsar *et al.*, 2011; Wei *et al.*, 2016; Adhyaru and Jacobson, 2018). Additionally, NLRP3 activation is implicated in neurodegenerative diseases, such as Alzheimer's disease, where amyloid-β plaques stimulate the inflammasome, leading to neuroinflammation and neuronal damage (Heneka *et al.*, 2012). Given its involvement in such a broad range of disorders, a deeper understanding of NLRP3 activation and regulation could lead to novel therapies with significant clinical benefits. While research on inhibitors targeting IL-1β, NLRP3, and caspase-1 is ongoing, the development of more specific therapeutics is crucial for advancing treatment strategies.

CAPS

Dysregulation or loss of NLRP3 has been extensively investigated and is linked to a variety of diseases and disorders, particularly inflammatory and autoimmune pathologies such as cryopyrin-associated periodic syndromes (CAPS) (Hoffman *et al.*, 2001; Moltrasio, Romagnuolo and Marzano, 2022). NLRP3 mutations are the primary cause of these rare systemic inflammatory disorders, which include three main syndromes: familial cold autoinflammatory syndrome (FCAS), characterised by rashes, fever, and joint pain induced by cold exposure; Muckle-Wells syndrome (MWS), a more severe condition that manifests in sporadic episodes unrelated to cold triggers; and neonatal onset multi-systemic inflammatory disease (NOMID), the most severe variant, which commences in infancy and results in chronic systemic inflammation (Mortimer *et al.*, 2016; Moltrasio, Romagnuolo and Marzano, 2022). All CAPS patients carry an autosomal dominant gain-of-function mutation in NLRP3 located on chromosome 1, with over 200 distinct mutations identified (de Menthière *et al.*, 2003). Mouse models carrying similar NLRP3 mutations reproduce the human symptoms, although some mutations in mice result in more severe and even lethal outcomes (Brydges *et al.*, 2009). While increased NLRP3 activity is linked to heightened inflammation, complete deficiency or non-functionality of NLRP3 is extremely rare and incompatible with survival In humans.

1.7 The absent in melanoma 2 inflammasome

The AIM2 inflammasome is triggered in response to double-stranded DNA (dsDNA) and protective against viral infections (Fernandes-Alnemri *et al.*, 2009b; Hornung *et al.*, 2009; Kumari *et al.*, 2020). AIM2 has recently been identified to have an enhanced response when exposed to increasing lengths of DNA with a minimum 70 base pairs of needed to activate, AIM2 and 200-base pairs being optimal (Sharma and de Alba, 2023). The AIM2 inflammasome belongs to the ALR family of receptors and contains an N-terminal pyrin domain and one or two C-terminal oligonucleotide binding domains which are connected by a long linker region (Wang and Yin, 2017). Within these C-terminals domains are two oligonucleotide binding folds, termed the HIN200 domains **(Fig 1.3)**, which contain positively charged arginine and lysine residues that allow for the negative charges associated with the phosphates in the DNA backbone (Roberts *et al.*, 2009; Jin *et al.*, 2012). The HIN200 domains alone are sufficient to bind to dsDNA, regardless of the rest of the AIM2 structure. Furthermore, multiple AIM2 proteins can cluster onto the same strand of dsDNA. This aids in the aggregation of the AIM2 inflammasome and assists in co-localisation and the formation of the full inflammasome. The

regulation of AIM2 is not fully understood; however, it is hypothesised that an autoinhibitory domain prevents activation in the absence of dsDNA (Jin *et al.*, 2013).

AIM2, which does not require a priming signal, acts as a sensor directly in the cytosol and, when triggered, induces the caspase-1 inflammasome as well as caspase-8, a potent apoptotic caspase (Pierini *et al.*, 2012). Because of the lack of specificity in its ligands, AIM2 has recently been implicated in autoimmune disorders, as it can sense not only pathogenic DNA but also self-DNA. This lack of specificity is hypothesised to play an important role in several autoinflammatory diseases such as psoriasis and systemic lupus erythematosus (Yang *et al.*, 2021; Yuxi Zhang *et al.*, 2023). Under homeostatic conditions, DNA is kept within the nucleus and protected from AIM2 sensing. However, there are numerous situations in which self-DNA migrates beyond this barrier and can trigger AIM2, leading to overreactive pyroptosis (Kumari *et al.*, 2020). This includes not only nuclear DNA but also mitochondrial DNA, which can be released into the cytosol during mitochondrial instability. This can occur when there is excessive accumulation of cholesterol and a deficit of its linked lipid 25-hydroxycholesterol (Dang *et al.*, 2017). In addition to mitochondrial DNA damage, AIM2 has been reported to activate in response to bacterial infections, such as *Listeria monocytogenes* and *Streptococcus pneumoniae* (Warren *et al.*, 2010). In these infections, the bacteria release their genetic material into the cytosol, thereby facilitating AIM2 activation. Finally, AIM2 plays an important role in neuronal development. Deficiency of AIM2 leads to altered morphology and behavioural changes in mice (Lammert *et al.*, 2020). Overall, AIM2 is a potent inflammasome with diverse functions. It provides protection against viral DNA and also helps prune and control abnormalities in cells.

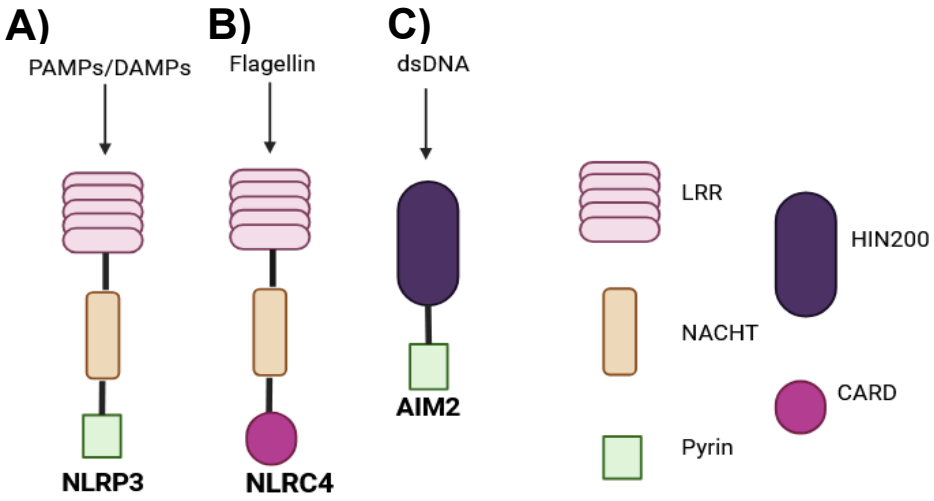


Figure 1.3 Structure of inflammasome sensor proteins.
A) NLRP3 is the most widely studied inflammasome sensor protein, responding to a wide range of activation signals. It consists of an LRR domain attached to a NACHT and a pyrin domain. **B)** NLRC4 has the simplest repertoire of responses among the most common inflammasome sensor proteins and shares a very similar structure with NLRP3. The main difference is that NLRC4 has a CARD domain attached to its NACHT domain instead of the typical pyrin domain. **C)** AIM2 functions intracellularly and targets dsDNA. It consists only of a HIN200 domain and a pyrin domain.

1.8 The NLRC4 inflammasome

NLRC4 is considered one of the most well-established inflammasomes, known for its defined trigger, activation of caspase-1, and induction of pyroptosis. This is due to its limited repertoire of ligands, responding only to the bacterial type III and type IV secretion systems, which are associated with the formation of pores in host membranes that allow the influx of virulence factors (Hayashi *et al.*, 2001; Yang *et al.*, 2013; Wen *et al.*, 2021). Originally thought to be a pro-apoptotic protein, NLRC4 has since been redefined and included in the inflammasome sensor protein family due to its structure (Duncan and Canna, 2018). As with all NLR family members, NLRC4 has three dominant structural domains: an N-terminal homotypic interaction domain, a central nucleotide-binding domain and a series of C-terminal LRRs (Hu *et al.*, 2013). NLRC4 does not bind directly to its ligands but instead works through an intermediary protein, NAIP, which binds to NLRC4-associated ligands and then to NLRC4 itself (Matico *et al.*, 2024). The identification of NAIP lends some strength to the hypothesis that NLRP3 does not directly bind its ligands but instead acts through an intermediary sensor protein. NLRC4 provides protection from bacterial infections, such as *Salmonella Typhimurium* and *Citrobacter rodentium*. However, it has been reported that NLRC4 knockout (KO) mice show no reduction in *S. Typhimurium* load (Broz, Newton, *et al.*, 2010). This is hypothesised to be due to the redundant protection provided by NLRP3 inflammasome, and only when both inflammasomes are absent do bacterial loads increase significantly compared to the control (Broz, Newton, *et al.*, 2010). Beyond its role in pathogenic infections, NLRC4 has been implicated in several autoinflammatory disorders, such as cold urticaria or ulcerative colitis(Romberg, Vogel and Canna, 2017a). The clinical outcomes for these diseases are improving as further studies on NLRC4 and the biomarkers associated with its malignant action are being identified, aiding in treatment (Romberg, Vogel and Canna, 2017b).

1.9 Lipids

Lipids are a diverse group of naturally occurring molecules that play essential roles in the structure, function, and regulation of living cells. These organic compounds, which include fats, oils, waxes, sterols, and phospholipids, are characterized by their insolubility in water and solubility in non-polar solvents (Munro, 2003; Wymann and Schneiter, 2008; Qiu *et al.*, 2023). Lipids are fundamental components of cellular membranes, providing structural integrity and flexibility through their role in forming the lipid bilayers that surround cells and organelles (Sunshine and Iruela-Arispe, 2017). Beyond their structural importance, lipids also serve as crucial energy reserves, stored in the form of triglycerides within specialised cells like adipocytes, where they can be broken down to meet the energy demands of organisms (Zadoorian, Du and Yang, 2023).

The functions of lipids, however, extend far beyond these classical roles. Recent research has revealed that lipids are not merely passive molecules but active participants in numerous cellular processes (Wymann and Schneiter, 2008). They play vital roles in signalling pathways, modulating a variety of biological processes including inflammation, metabolism, and cell communication (Downes and Currie, 1998; Leroux *et al.*, 2012; Natesan and Kim, 2021). Lipids function as signalling molecules themselves, influencing the behaviour of immune cells and regulating metabolic pathways, and they serve as precursors to bioactive compounds like hormones and vitamins. This multifaceted nature of lipids makes them key players in maintaining cellular homeostasis and responding to changes in the internal and external environment.

1.10 Lipid biosynthesis

1.10.1 Transcriptional regulation of lipid biosynthesis

Many lipids serve as signalling molecules that are crucial for various biological processes, making them essential for maintaining homeostasis. Cells typically do not need to synthesise the building blocks for most lipids, as these components are readily available from exogenous sources. Instead, cells can modify these lipids to meet their specific requirements through a series of elongation or reduction reactions (Florance and Ramasubbu, 2023). This ability to uptake and use external lipids allows for the maintenance of homeostatic conditions when all dietary requirements are satisfied; however, when exogenous lipids are not abundant, lipids must be produced endogenously. In such conditions, the activation of the sterol regulatory element binding proteins (SREBPs) is necessary (DeBose-Boyd and Ye, 2018). SREBPs act as cholesterol synthesis regulatory membrane-bound transcription factors and are involved in

the production of a broad number of lipid biosynthesis genes (Ye and DeBose-Boyd, 2011). Made of a tripartite structure consisting of an NH_2-terminal transcription factor domain, a COOH-terminal regulatory domain and a middle hydrophobic linker region that has two hydrophobic transmembrane segments, these transcription factors are bound to the endoplasmic reticulum (Brown and Goldstein, 1997).

To prevent unwanted activation of these master transcription factors, SREBP is kept in a regulatory complex with insulin-induced gene proteins (INSIG) and chaperon SCAP (Shimano and Sato, 2017). INSIG-1, and recently identified INSIG-2, act to block the movement of the SCAP-SREBP complex by facilitating the ability of the complex to remain in the ER until the cells face sterol depletion, at which point the SCAP-SREBP complex is released (Yang *et al.*, 2002). SCAP is a membrane-bound protein with two domains, an N-terminal domain with alternating hydrophobic and hydrophilic sequences, and a C-terminal hydrophilic domain with a highly conserved sequence which is suspected to allow for SCAP to interact with a wide variety of signalling molecules such as G proteins (Brown and Goldstein, 1997). Upon the detection of sterol depletion by INSIG, the SCAP-SREBP complex is released from the ER. Once released the complex translocates to the Golgi where it interacts sequentially with site 1 (S1P) and site 2 protease (S2P) respectively.

These proteases cleave the SREBP at the SCAP binding site and part of the N-terminus. This cleavage is essential and dependent on positively charged residues in the SREBP sequence, which direct the site's protease function. Loss of these positive charges completely inhibits SREBP signalling (Brown and Goldstein, 1997). Once S1P has broken the covalent bond between the two transmembrane domains of SREBP, both segments are bound to the ER membrane. Only when S2P cleaves the N-terminal domain, SREBP enters the cytosol and move to the nucleus via a nuclease localisation signal. Cleavage by S1P is tightly regulated by sterols and does not occur in an abundant state (DeBose-Boyd *et al.*, 1999). S2P are dependent on S1P but are not sterol-regulated (Guo *et al.*, 2018). Upon translocation to the nucleus, the transcription factor SREBP binds to appropriate sterol response element (SRE) sites in the target genes regulating their transcription.

It is important to note that there are three distinct isoforms of SREBP with overlapping but distinct roles. SREBP-2 is primarily involved in cholesterol synthesis whereas SREBP-1a and SREBP-1c are associated with fatty acid synthesis (Hannah *et al.*, 2001). Whilst there are three isoforms, SREBP-1a and SREBP-1c are encoded by the same gene on human chromosome 17. An alternative transcription start site allows for the production of these two distinct isoforms, whereas SREBP-2 is encoded on chromosome 22 (Amemiya-Kudo *et al.*,

2002). SREBP-1c is the least active of the three SREBPs, with SREBP-1a and SREBP-2 being the predominant isoforms in most cells (Brown and Goldstein, 1997). It is also important to note that when expressed at higher physiological levels, there is a significant degree of redundancy, with SREBP-1a/c and SREBP-2 being able to activate genes in the corresponding pathways (Brown and Goldstein, 1997). Recent research has implicated SCAP-SREBP translocation in NLRP3 activation, suggesting that this lipid regulation pathway can aid in the spatial localisation of NLRP3 (Guo *et al.*, 2018).

1.10.2 Cholesterol biosynthesis

One of the most important lipid pathways is cholesterol biosynthesis, a complex process that not only produces cholesterol but also generates various intermediates essential for cellular function. Cholesterol is a critical structural component of cell membranes, contributing to membrane fluidity and integrity. It also serves as a precursor for the synthesis of steroid hormones, bile acids, and vitamin D (Feingold and Grunfeld, 2000; Maxfield and van Meer, 2010; Barritt, DuBois-Coyne and Dibble, 2024). The cholesterol biosynthesis pathway involves multiple enzymatic steps, beginning with the conversion of acetyl-CoA to mevalonate. This rate-limiting step is catalysed by HMG-CoA reductase and progresses through a series of intermediates to produce cholesterol (Istvan and Deisenhofer, 2001).

Beyond its structural and metabolic roles, cholesterol and its intermediates have been linked to immune function, particularly in the regulation of the NLRP3 inflammasome. Cholesterol accumulation in cellular membranes, especially within lipid rafts, has been shown to facilitate the assembly of the NLRP3 inflammasome (Helms and Zurzolo, 2004). For instance, the formation of intracellular cholesterol crystals can destabilise the lysosomal membrane, which has been shown to induce NLRP3 activation (Duewell *et al.*, 2010b). Furthermore, oxidised LDL, a lipid known to accumulate in blood vessels, is absorbed by macrophages and forms crystals within the lysosome (Duewell *et al.*, 2010b). Oxidised LDL has also been shown to activate TLR4/6, suggesting that it may provide both the priming and activation signals required for NLRP3 inflammasome activation (Kumar *et al.*, 2020). ATP-binding cassette transporter A1 (ABCA1) has been shown to promote cholesterol efflux, and its absence leads to cholesterol accumulation and enhanced TLR4-mediated pro-inflammatory signals (Adorni *et al.*, 2007). Furthermore inhibition of NPC1 cholesterol transport and efflux from lysosomal compartments, has been demonstrated to dampen NLRP3 activation (de la Roche *et al.*, 2018). Beyond cholesterol itself, certain intermediates of the cholesterol biosynthesis pathway, such as geranylgeranyl pyrophosphate, have been implicated in the priming phase of NLRP3 activation (Lordén *et al.*, 2017). This dual role of cholesterol and its biosynthetic intermediates

positions the pathway as a key regulator of inflammation and immune responses, making it a potential target for therapeutic intervention.

1.10.3 Fatty acid biosynthesis

Fatty acids are key building blocks of lipids, consisting of long hydrocarbon chains with a carboxylic acid group at one end. They play crucial roles in cellular functions, serving as components of phospholipids and glycolipids, which form cell membranes and contribute to membrane fluidity and integrity (Swinnen *et al.*, 2003; Sunshine and Iruela-Arispe, 2017). It is well known that fatty acids are a significant energy source, as they can be broken down through β-oxidation to generate ATP, making them essential for energy metabolism. Additionally, certain fatty acids act as precursors for bioactive molecules like prostaglandins and other signalling lipids, which regulate processes such as inflammation, immune response, and cellular communication (Bayer and Föster, 1979; Hata and Breyer, 2004). Their structural, energetic, and signalling roles make fatty acids indispensable for maintaining cellular health and function. Essential for the production of fatty acids is the enzyme fatty acid synthase (FASN).

FASN is a holoenzyme produced in response to sterol depletion by SREBP and leads to the production of long chain fatty acids via a series of elongation reactions initiated with short chain fatty acids (Chi *et al.*, 2022). These long-chain saturated fatty acids act as crucial components for the building of lipid microdomains, allowing for correct localisation and activation of receptors, as well as the as energy storage molecules (Munro, 2003; Swinnen *et al.*, 2003). An offshoot of FASN is the ability to produce PIP4 and lysophosphatidic acid, which regulate the PI3K/AKT pathway and G protein-coupled receptors essential in mitosis (Klarić *et al.*, 2020).

The FASN enzyme is composed of two identical multifunctional polypeptides with 7 sites of catalytic activity (Maier, Leibundgut and Ban, 2008), transcribed by the *FASN* gene and its expression is induced by SREBP upon sterol depletion. Once produced, the polypeptides bind together and catalytically convert acetyl-CoA and malonyl-CoA to palmitate via a series of elongation reactions. The initial reactants, that is the acetyl-CoA and malonyl-CoA, are produced from citrate and the TCA cycle in mitochondria. This reaction is dependent on the presence of the initial reactants as well as NADPH as a source of reducing equivalents. It is classified as a decarboxylative class condensation reaction with the FASN enzyme being responsible for condensation, transacylation, reduction and dehydration (Xiao *et al.*, 2024). Interestingly FASN can also use two acetyl-CoAs rather than the classic pathway of an acetyl-

CoA and malonyl-CoA to produce palmitate. The dual acetyl-CoA method of lipid production is less energy-efficient with FASN favouring malonyl-CoA with acetyl-CoA (Nelson and Cox, 2008). However, the use of two acetyl-CoA to produce acetoacetyl-CoA is an initial stage of the cholesterol pathway, suggesting that the malonyl-CoA with acetyl-CoA to produce acetoacetyl -CoA could be a more favourable and rapid start point for the cholesterol biosynthesis pathway (Nelson and Cox, 2008). Furthermore FASN can utilise methylmalonyl-CoA instead of malonyl-CoA as its starting reactant, which causes the production of methyl-branched fatty acids to be produced (Dewulf *et al.*, 2019).

The structure of FASN has been solved with two distinct organizations of highly conserved catalytic domains within the multi-enzyme structure. In the mammalian FASN there are three catalytic domains in the N-terminal section, with a ketoacyl synthase (KS) malonyl/acetyltransferase (MAT) and dehydratase (DH) domain followed by a linking core region connecting to the C-terminal domain which contains enoyl reductase (ER), ketoacyl reductase (KR) acyl carrier protein (ACP) and thioesters (TE) domains (Maier, Leibundgut and Ban, 2008). The interdomain allows for the connection of the two homodimers to form the complete FASN complex (Maier, Leibundgut and Ban, 2008) (**Fig 1.4, 1.5**) In regular homeostasis, FASN is expressed when there is a need for lipid biosynthesis.

FASN expression is critical for early embryo development and deletion is fatal, as FASN activity is essential for foetal lung development (Rooney, 1989). Whilst expressed in most tissues, FASN is often not active due to the dietary abundance of palmitate, the most prevalent saturated fatty acid in the human body (Carta *et al.*, 2017). The exception is the liver where FASN is a housekeeping protein for normal hepatic triglyceride mechanisms and high activity in the lung, breast, adipose and brain tissue has also been observed (Fhu and Ali, 2020). Within cells, FASN is typically found localized on the plasma membrane or cytosol, where it can come into contact with ligands easily (Xiao *et al.*, 2024).

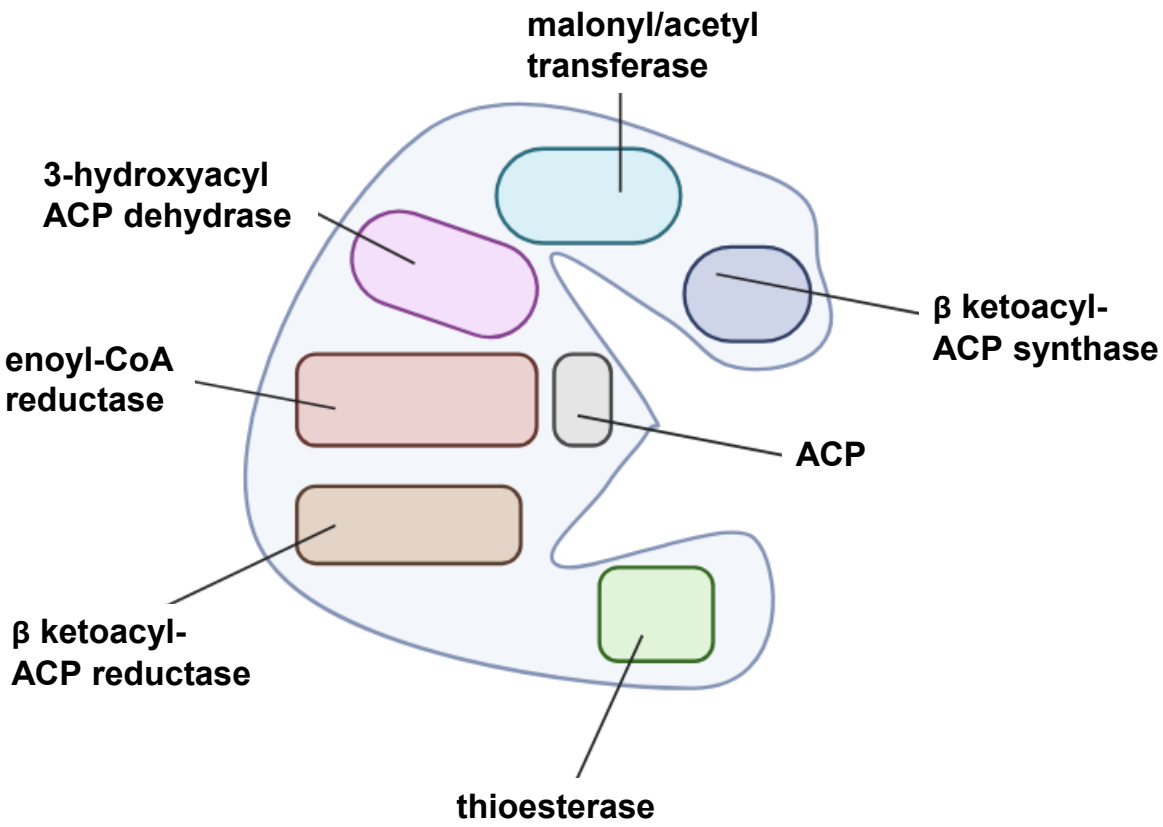


Figure 1.4: Structure of the FASN enzyme

Depiction of an FASN monomer showing the active sites where precursor lipids are modified for the synthesis of long-chain fatty acids. Each site interacts as described in the text, with ACP serving as a shuttle, facilitating the transport of lipids to different active sites of the enzyme. FASN is inactive in its monomeric form and requires dimerization to become functional. Created with BioRender.com, based on (Ahern, Kevin. Indira, 2021)

FASN has been identified as a biomarker for the severity of cancer development, with the identification of FASN activity being used to mark prognosis (Fhu and Ali, 2020). As fatty acids are necessary molecules for all lipid metabolism, it's essential that cancer cells can gain control of the means of production and enable aberrant de novo lipid synthesis. In cancer cells, malignant levels of FASN are produced via upregulation of the FASN machinery by cancer genes (Flavin *et al.*, 2010). Beyond its role in oncology, FASN has recently been implicated in having a crucial role in inflammasome priming due to the production of acetoacetyl-CoA and its necessity in forming lipid rafts in the plasma membrane for TLR signalling (Carroll *et al.*, 2018). Whilst this is one crucial function of FASN, its multiple domains and the fact it produces such vital lipids suggest it has more potential functionality.

During metabolic stress, FASN expression is upregulated, driving fatty acid metabolism and contributing to inflammasome activation in macrophages. Inhibition of FASN, particularly in combination with metformin, suppresses LPS-induced proinflammatory responses in macrophages via AKT signalling and has been shown to impact inflammasome priming (Xiong et al., 2021). Beyond its role in priming, FASN also contributes to immune signalling by modulating the TLR4 pathway and influencing downstream cytokine production through sphingolipid synthesis, which affects membrane raft formation and Rho-GTPase trafficking (Du et al., 2024). In macrophages, FASN is emerging as a key regulator of inflammation through its involvement in metabolic transitions between resting and activated states.

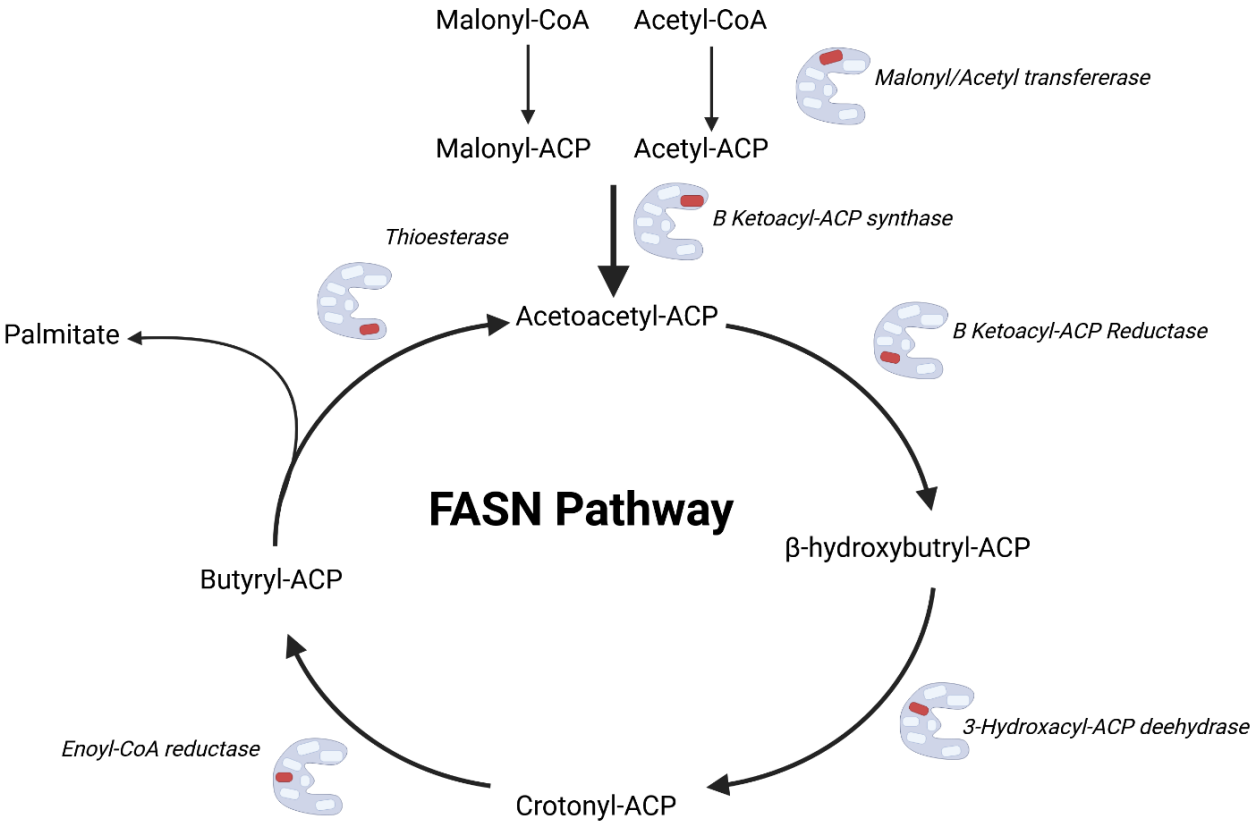


Figure 1.5 FASN pathway.
Pathway illustrating the biosynthesis of palmitate by FASN. The process begins with either malonyl-CoA and acetyl-CoA or two acetyl-CoA, though the latter is less efficient. These lipids, derived from the TCA and citrate cycles, undergo a series of enzymatic reactions to produce palmitate. Each reaction requires activation of a specific site within the enzyme (Carroll *et al.*, 2018).

1.10.4 Fatty acid synthesis and translational research

Due to its upregulation in cancer and the increasing awareness of lipid-related diseases, research has focused on inhibiting the FASN pathway. In recent years, it has become apparent that whilst the targeting of FASN is important to disease, the modulation of FASN activity is also essential for the survival, differentiation, and function of various immune cells. Macrophages have been associated with FASN due to its ability to induce polarisation, influence chemotaxis and influence the bactericidal function of macrophages (Mesquita *et al.*, 2020; Xiong *et al.*, 2021). With its activity in macrophages, FASN has also been implicated in the formation of ox-LDL-induced macrophage foam cells, which contribute to the pathogenesis of atherosclerosis (Kumar *et al.*, 2020). This implication in disease as well as its role in cancers means that the inhibition of FASN is an area of important translation research.

The small molecule inhibitor cerulenin is a natural product of *Cephalosporium caerulein* fungus found occurring in nature and was identified to be selectively toxic to cancer cells *in vitro*. It works via covalently binding to the active site of cysteine thiol in the beta-ketoacyl-synthase domain irreversibly inhibiting FASN function (Moche *et al.*, 1999). Whilst significantly able to inhibit FASN, cerulenin was deemed too unstable as a systemic anti-cancer agent. To try and mitigate this instability, a more stable derivative, C75, was developed (Kuhajda *et al.*, 2000). Functionally identical to cerulenin, this small molecule inhibitor has been shown to increase malonyl-CoA accumulation, an indicator of FASN inhibition, with an indirect effect on inhibiting CPT-1 activity (Mera *et al.*, 2009). Both C75 and cerulenin show promise for translational applications, despite being unsuitable as anti-cancer agents due to their association with weight loss, a symptom commonly linked to cancer progression (Flavin *et al.*, 2010).

1.11 Palmitate

Palmitate is the most abundant saturated fatty acid making up to 20-30% of total fatty acids in humans (Carta *et al.*, 2017). In typical homeostatic conditions, palmitate is supplied from dietary sources and does not need to be produced via the FASN enzyme activation. Due to its vital role, palmitate is tightly balanced with a depletion in the dietary supply being supplemented by an increase in lipid biosynthesis (Saponaro *et al.*, 2015). Whilst production is balanced there are also mechanisms in place to prevent accumulation through the desaturation to simpler fatty acids such as oleic acid or its elongation to more complex fatty acids such as stearic acid (Bruce and Salter, 1996). This means that the total cellular palmitate supply fluctuates during homeostasis. It is important to note that palmitate can exist in its salt form or ester form as palmitic acid, with both sharing the same fatty acid chain but differing in their ionisation state. Palmitic acid is typically present only transiently or extracellularly, whilst

palmitate is biologically relevant, being able to interact with enzymes, transporters and membranes (Carta *et al.*, 2017). High levels of palmitic acid in the extracellular milieu have additionally been shown to enhance the transition of macrophages to an inflammatory phenotype, which are more inclined to undergo caspase-1 activation (Qiu *et al.*, 2023). The role of palmitate and its function is not fully understood in the immune context, but its high abundance and versatility make it an attractive target for research.

Tight homeostatic regulation ensures controlled palmitate production. However, excessive levels of palmitate or palmitic acid can contribute to pathogenesis. Elevated palmitate production has been associated with atherosclerosis, neurodegenerative diseases, and cancer, independent of dietary intake (Carta *et al.*, 2017). When overproduced, palmitate results in systemic inflammatory responses with metabolic dysregulation, dyslipidaemia, insulin resistance and a dysregulated fat deposition and distribution (Murru *et al.*, 2022). Recently, it has been identified that palmitic acid has a direct interaction with the inflammasome, through TLR4 signalling pathway in hepatic stellate cells (Dong *et al.*, 2020).

1.12 Palmitoylation

While many PTMs have been studied, palmitoylation—the addition of palmitate—has recently emerged as a significant focus in PTM research (Draper, Xia and Smith, 2007). This modification is associated with protein stabilisation by preventing ubiquitination. It also increases protein hydrophobicity, facilitating membrane attachment and thereby influencing protein localisation and function (Linder and Deschenes, 2007a). Belonging to the subtype of fatty acid modifications called S-acylation palmitoylation, or *S*-palmitoylation, this PTM involves the attachment of palmitate to the free thiols typically found in cysteine residues (Fukata, Bredt and Fukata, 2006). It should be noted that palmitoylation also infrequently occurs on serine and threonine residues and is termed O-palmitoylation. Regulated by the family of zinc finger palmitoyl transferase enzymes, palmitoylation has been found to have functional significance, allowing for proteins to be membrane-bound, alter stability for protein-protein interactions and act to regulate protein trafficking (Tabaczar *et al.*, 2017). Palmitoylation is unique in the family of lipid PTMs as it is highly dynamic and readily reversible, due to the lability of the thioester bond (Guan and Fierke, 2011).

Palmitoylation has long been implicated in the regulation of immunity and inflammation, with proteins associated with PRR signalling and cytokine production being implicated in innate immunity and TCR and B-cell antibody production (Ladygina, Martin and Altman, 2011; Zhang *et al.*, 2021). The relationship between a protein and its palmitoylation state is not constant, and proteins are likely to undergo multiple cycles of palmitoylation and de-palmitoylation within

their lifetime unless they are tightly regulated to prevent aberrant palmitoylation (Liu *et al.*, 2022).

1.13 Immunometabolism

Immunometabolism is emerging as a critical area in understanding how the inflammasome operates, particularly in the context of the NLRP3 inflammasome. This field explores the intricate relationship between metabolic processes and immune responses, shedding light on how various metabolites and energy states can influence the activation and regulation of immune pathways. While the metabolic regulation of NLRP3 activation has been extensively studied, many aspects remain unclear and warrant further investigation.

1.13.1 Glycolysis

Glucose metabolism is essential for maintaining homeostatic conditions. However, a high-glucose state has been shown to increase the expression of E74-like ETS transcription factors and decrease methyltransferase activity, leading to an increase in MARK4 expression (Wang *et al.*, 2020). This pathway has been shown to induce the NLRP3 inflammasome through microtubule-dependent mechanisms (Li *et al.*, 2017). Pyruvate is a key intermediary metabolite and the end product of glycolysis, transforming into acetyl-CoA, lactate or alanine. Pyruvate has been implicated in inhibition of NLRP3, preventing ATP- or nigericin-triggered release of mtDNA into the cytoplasm (Li *et al.*, 2018). An alternative enzyme involved in glycolysis is hexokinase 1 which facilitates glucose entry into glycolysis in the cytosol. This enzyme has been implicated to aid in inflammasome activation and even hypothesised to be its own PRR leading to NLRP3 activation (Wolf *et al.*, 2016).

Cytosolic accumulation of glucose 6-phophsate (G6P) elevates NLRP3 activation and is facilitated by glucose-6-phosphase dehydrogenase (G6PD), which catalyses the entry of glucose to the pentose phosphate pathway (H. C. Yang *et al.*, 2019). When inhibition of G6P was induced, there was increased susceptibility to *Staphylococcus aureus* and *Escherichia coli* (Yen *et al.*, 2019). This was further validated by showing that inhibition of glycolytic enzymes GAPDH and α-enolase, with small molecule GB111-NH2 leads to inflammasome activation via glycolytic flux (Sanman *et al.*, 2016).

1.13.2 TCA cycle

Distinct from glycolysis, the TCA cycle has been implicated in NLRP3 inflammasome regulation. TGF-B1 has been linked to succinate accumulation, via the inhibition of succinate dehydrogenase activation (Williams and O'Neill, 2018), which has also been implicated to

induce NLRP3 activation (Harber *et al.*, 2020; Hooftman *et al.*, 2020). In contrast, interestingly, citrate-derived itaconate has been explored as a potential anti-inflammatory modulator in macrophages, acting via alkylation of Cys548 and prevention of NEK7 binding (Mills *et al.*, 2018).

Among other components of the TCA cycle, fumarate has been implicated in the immunometabolic regulation of NLRP3. Fumarate, an intermediate in the citric acid cycle, can block GSDMD processing, oligomerization, and pyroptosis. It was demonstrated that Cys192 in mice and Cys191 in humans are required for GSDMD processing by caspase-1 and that these sites can undergo succination. However, in the presence of fumarate GSDMD succination was reduced (Harber *et al.*, 2020). Fumarate similarly blocked GSDME, which is associated with apoptosis in mice lacking GSDMD (Rogers *et al.*, 2019).

Regulation of metabolism is crucial for pathogens to persist within host cells. Metabolites from the TCA cycle are essential for the survival of *Pseudomonas aeruginosa* and *Mycobacterium tuberculosis*, as observed in various murine models of lung infection (McKinney *et al.*, 2000; Lindsey *et al.*, 2008). It has been suggested that the regulation of inflammasomes by TCA metabolites may also support the persistence of chronic infections (Traven and Naderer, 2019; Rosenberg *et al.*, 2021). Therefore, while the immunomodulatory effects of TCA metabolites could offer therapeutic potential for treating NLRP3-related sterile inflammatory conditions, such as rheumatoid arthritis, they might also impair host immune responses and hinder pathogen clearance, though this remains speculative.

1.13.3 Fatty acids

Saturated fatty acids (SFAs) have been shown to activate the NLRP3 inflammasome, while monounsaturated fatty acids (MUFAs) play a critical role in counteracting this activation and downregulating NLRP3 expression (Wen *et al.*, 2011a; L'homme *et al.*, 2013; Huang *et al.*, 2022). The relationship between these fatty acids and NLRP3 is further complicated by fatty acid oxidation (FAO), which occurs in the mitochondria through β-oxidation. This process produces acetyl-CoA, feeding into the TCA cycle and generating ATP via oxidative phosphorylation (OXPHOS). Carnitine palmitoyltransferase 1A (CPT1A) -mediated FAO has been shown to influence NLRP3 inflammasome activity in Hif2a-deficient macrophages, contributing to improved insulin sensitivity in obese mice (Li *et al.*, 2021). Additionally, blocking FAO in macrophages can slow the progression of atherosclerosis and gouty inflammation in animal models (Hall *et al.*, 2018; Hohensinner *et al.*, 2021).

1.14 FASN as a regulator of NLRP3

Previous studies have established a connection between FASN and the NLRP3 inflammasome, highlighting the role of FASN in inflammasome priming. Initial research demonstrated that the product of FASN, palmitate, can induce inflammasome activation, suggesting that long-chain saturated fatty acids, when present for prolonged periods, are capable of triggering this process (Wen *et al.*, 2011a). Subsequent studies further explored this relationship, hypothesising that FASN is crucial for TLR-mediated membrane raft signalling. One study indicated that Rho-GTPase activity, and subsequently NLRP3, was disrupted upon FASN knockout, while another proposed that the intermediate product of FASN, acetoacetyl-CoA, serves a dual function in both inflammasome activation and the cholesterol biosynthesis pathway (Wei *et al.*, 2016; Carroll *et al.*, 2018). Both studies concluded that it was cholesterol, rather than palmitate or FASN directly, that influenced NLRP3 activation. This hypothesis was further supported by later research investigating the drug metformin, which dampens FASN activity and was shown to inhibit NLRP3 activation (Xiong *et al.*, 2021). However, all previous work has predominantly focused on LPS-induced priming of NLRP3, with particular emphasis on the upstream events leading to NLRP3 expression. While this is essential, it offers limited insight into the precise regulation of NLRP3 during activation. Recent studies have highlighted that the journey of NLRP3 from transcription to inflammasome formation is tightly controlled (Chen and Chen, 2018b; Lee *et al.*, 2023; Zhang. *et al.*, 2023), so it is prudent to investigate the relationship between NLRP3 and FASN beyond the established connection during priming.

1.15 Aims and Objectives

The NLRP3 inflammasome is one of the most revolutionary discoveries in innate immunity, and its ability to act as a sensor of disruption in homeostatic conditions make it an interesting target for therapeutics. However, precisely how this potent inflammatory response is regulated remains unknown. Recent studies have shown pivotal roles for lipid metabolism in NLRP3 inflammasome activation. For example, upon sensing an activation signal, NLRP3 must translocate to TGN38-positive vesicles which are rich in PI4P, an acidic phospholipid that helps NLRP3 anchor to membranes. However, a comprehensive understanding of lipid metabolism, specifically fatty acid synthesis, in NLRP3 activation is lacking. We also do not know the mechanistic details that aid NLRP3 tether to TGN38-positive vesicles. This study aims to address two related questions:

Part 1

Fatty acid synthesis is a vital process for the *de novo* synthesis of fatty acids and is profoundly dependent on a multi-functional enzyme FASN. The enzyme utilizes metabolic substrates to synthesize new fatty acids, which play key roles in structural integrity of membranes and in energy homeostasis. Previous studies have suggested a role for fatty acid synthesis in NLRP3 priming. However, its' role in inflammasome activation remains unknown. Here, I aim to examine the role of fatty acid synthesis in NLRP3 inflammasome activation by specifically targeting the FASN enzyme in the pathway.

Objectives:

- Investigate whether fatty acid synthesis regulates NLRP3 activation, independently of the role of FASN in inflammasome priming.
- Examine whether the above response is specific to the NLRP3 inflammasome.
- Dissect mechanistic details by which FASN regulates NLRP3 inflammasome activation.

Part 2

The lipidome, encompassing the full spectrum of lipids within a biological system, is being increasingly recognised as a key regulator of cellular processes. Beyond serving as an energy reservoir, the lipidome provides structural components for cellular architecture and bioactive molecules that influence immune pathways. These multifaceted roles highlight the ability of lipidome to influence cellular, metabolic, and immune activities. The full spectrum of changes in lipidome during NLRP3 inflammasome activation are not known.

Objectives:

- Utilize advanced techniques in mass spectrometry to gain a deeper understanding of the lipidome during NLRP3 inflammasome activation.
- Decipher novel lipids involved in the priming and activation phase of the NLRP3 inflammasome.
- Compare and identify distinct lipids involved during canonical and non-canonical NLRP3 inflammasome activation.

Chapter 2

Material and methods

Chapter 2. Material and Methods

2.1 Reagents Table

Table 2.1 - Culture Reagents

Cell Culture Media	Ingredients	Source/Product number
Complete DMEM	DMEM FBS (10%, heat inactivated) Penicillin/Stretomycin (1%) Hepes buffer (1%) L929-cell supernatant (10%)	Sigma (D6429) Sigma (F7524) Sigma (P0781) Lonza (17-737E) L929-cells were kindly gifted from Jacques Behmoaras, Imperial College London
BMDM media	DMEM FBS (10%, heat inactivated) Penicillin/Stretomycin (1%) Hepes buffer (1%)	Sigma (D6429) Sigma (F7524) Sigma (P0781) Lonza (17-737E)
RPMI	FBS (10%, heat inactivated) Penicillin/Stretomycin (1%) Hepes buffer (1%)	Sigma (F7524) Sigma (P0781) Lonza (17-737E)
Opti-MEM	-	Thermo Fisher - 31985062

Table 2.1 - Commercial Reagents

Reagent	Source/ Product number
LPS from E. coli 055:B5	Invivogen (tlrl-b5lps)
Pam3CSK4	(tlrl-pms)
Poly(dA:dT)	(tlrl-patc)
ATP	Sigma (A1852)
Chloroform	(C2432)
Ethanol	(51976)
Isopropanol	(I9516)
LB broth	(L3022)
LB broth with agar	(L2897)
Streptavidin agarose	(S1638)
N-ethylmaleimide (NEM)	(E3876)
Hydroxylamine	(438227)
2 Bromohexadecanoic acid (2-BP)	(21604)
Propidium Iodide	(P4170)

Pierce BCA Protein Assay Kit TNF-α ELISA kit IL-1β ELISA kit Lipofectamine® 2000 Pierce™ LDH Cytotoxicity Assay Kit	ThermoFisher (23225) (88-7324-22) (88-7013-22) (11668027) (88953)
Biotin-HDPD	Abcam (ab145614)
Cerulenin AM-FLICA Caspase-1 (YVAD)	Enzo (LKT-C1869-M001) (CT-98)
C75 GSK2194069 Orlistat	Cayman Chemical (CAY10005270) (CAY20022) (CAY10005426)
BSA-Palmitate	Merck (90951)

Table 2.3 - Software

Program	Supplier	Link
Image J	National Institutes of Health	https://imagej.net/
Prism	GraphPad	https://www.graphpad.com/
GPS-Palm	The Cuckoo Workgroup	https://gpspalm.biocuckoo.cn/

Table 2.4 -Plasmids

Plasmid	Source/ Product number
pLV-CMV-NLRP3-Hygro pcDNA3-N-Flag-caspase-1 pcDNA3-N-Flag-ASC1	Addgene #176056 #75128 #75134
pBEN- NLRP3 N-Flag pBEN-caspase-1 C-Myc pBEN-ASC N-Myc	Inflammasome reconstitution plasmids were a kind gift from Prof. Thomas Miethke
mouse caspase-1 plasmid mouse ASC plasmid	Mouse inflammasome plasmids were a kind gift from Bruce Beutler
Myc-NLRP3 WT, C671A, C835A, C898A	MRC PPU Reagents and Services facility, Dundee

2.1 Ethics Statement

All experiments were conducted in compliance with the Animals (Scientific Procedures) Act 1986, under the appropriate UK Home Office license, and following established institutional guidelines.

2.2 Mice

Wild-type (WT) C57BL/6J mice were sourced from Charles River, UK. All animal procedures were conducted under appropriate personal and project licences (PP4111881), in compliance with the Animals (Scientific Procedures) Act 1986. These procedures were approved by the Imperial College Animal Welfare and Ethical Review Body (AWERB) and the UK Home Office. For in vitro experiments, both male and female mice were used in approximately equal proportions, with ages ranging from 8 to 12 weeks.

2.3 Macrophage differentiation

Immortalised bone marrow-derived macrophages (iBMDMs) were maintained in DMEM containing 10% heat-inactivated FBS and 1% penicillin/streptomycin. Cells were cultured in 10 cm² dishes and split every 2–3 days upon reaching 90% confluency. *Gsdmd*$^{-/-}$ iBMDMs were generously provided by Feng Shao through A. Shenoy. Primary mouse bone marrow cells were isolated from the femurs and tibias of 8–12-week-old C57BL/6 mice and cultured in L929-conditioned media. To generate bone marrow-derived macrophages (BMDMs), bone marrow cells were differentiated at 37°C over 5–6 days in L929-conditioned DMEM supplemented with 10% heat-inactivated FBS, 1% penicillin/streptomycin, and 1% HEPES (Table 2.1). Human monocyte-derived macrophages (hMDMs) were isolated from de-identified buffy cones using gradient separation with Histopaque 1077. Monocytes were differentiated over five days in RPMI supplemented with 100 ng/mL macrophage colony-stimulating factor (M-CSF) and 10% FBS.

2.4 Canonical NLRP3 inflammasome activation

Primary BMDMs, iBMDMs, and hMDMs were seeded in 24-, 12-, or 6-well plates at densities of 0.5×10^6, 1.0×10^6, or 2.5×10^6 cells per well, respectively, and allowed to adhere overnight. To activate the NLRP3 inflammasome, cells were treated with LPS (500 ng/ml) or Pam3CSK4 (500ng/ml) for 4 hours, followed by ATP (5 mM) or Nigericin (20 nM) for 60 minutes (**Table 2.2**). Where noted, cerulenin and C75 were added 1 hour prior to ATP stimulation, or simultaneously with LPS or ATP as indicated. 2-bromohexadecanoic acid (2-BP) was added either 2 hours before ATP or simultaneously with LPS or ATP. Palmitate was first conjugated

to fatty acid-free BSA before addition to the cell culture media, and supplemented to media at a 100 μM concentration 24 hours prior to inflammasome activation.

2.5 Non-canonical NLRP3 inflammasome activation

Macrophages were seeded in 12-well plates at a density of 1×10^6 cells per well. The following morning, cells were gently washed with PBS, and 500 μL of fresh DMEM was added to each well. Cells were rested for approximately 30–60 minutes prior to the addition of Pam3 (1 μg/mL) for a 3-hour stimulation. Following Pam3 treatment, macrophages were transfected with LPS: 2.5 μg of LPS was diluted in 50 μL of OPTI-MEM, and 0.75 μL of Lipofectamine 2000 was separately diluted in another 50 μL of OPTI-MEM. The diluted Lipofectamine 2000 was added dropwise to the diluted LPS solution, gently vortexed, and incubated for 15 minutes to allow complex formation. The LPS-Lipofectamine complex was then added dropwise to the cells (1ug/ml), which were incubated for 16–20 hours. Supernatants and cell lysates were then collected as previously described.

2.6 NLRC4 stimulation

Salmonella typhimurium was cultured overnight in 5 mL Luria-Bertani (LB) broth at 37°C with shaking at 220 rpm. The following day, a sub-culture was prepared by adding 500 μL of the overnight culture to 5 mL fresh LB broth, followed by incubation for approximately 1 hour to achieve an optical density (OD) between 0.4 and 0.8. The sub-culture was centrifuged at 3000 x g for 10 minutes, the supernatant was discarded, and the pellet was resuspended in 5 mL PBS for OD measurement. Bacteria were then added to the designated cell types at a multiplicity of infection (MOI) of 2 and incubated for 4–5 hours. Supernatants and cell lysates were collected as described above

2.7 AIM2 inflammasome

iBMDMs or BMDMs were seeded at a density of 0.5×10^6 cells per well in a 24-well plate with complete media. Where indicated, cells were stimulated with LPS (500 ng/mL) for 2 hours prior to DNA transfection. To prepare the transfection complex, 1 μL of Lipofectamine 2000 was diluted in 25 μL of OPTI-MEM media, and 1 μg of Poly(dA:dT) was diluted separately in 25 μL of OPTI-MEM. The solutions were mixed by vortexing and incubated at room temperature for 15–20 minutes to allow complex formation. The Poly(dA:dT)/Lipofectamine complexes were then added dropwise to the BMDMs/iBMDMs, followed by incubation for approximately 4 hours. Cell lysates and supernatants were collected as described above.

Table 2.5 - Buffers

Buffer	Composition
Cell lysis buffer	1ml Triton-X 100 200 µl DTT Protease inhibitor (1 tablet) 330 µl of 1.5M Tris-cl Water to 10 ml
RIPA lysis buffer	1 ml Triton-X 100 0.5 g Sodium deoxycholate 1ml or 10% SDS 0.876 g NaCl 3.3 ml of Tris-Cl (pH 8.8) Water to 100 ml (Protease inhibitor and Phosphatase inhibitor added to each 10ml aliquot)
Immunoblotting reagents (All reagents obtained from Sigma)	Running Buffer (10X) • 30 g Tris Base • 144 g Glycine • 10 g SDS • Water to 1000 ml

	Transfer Buffer (10X) • 30.3 g Tris Base • 144 g Glycine • Water to 1000 ml 10X TBS (pH 7.6) • 80 g NaCl • 24.2 g Tris Base • pH to 7.6 with HCl • Water to 1000 ml
KPBS buffer	25 mM KCL 100 mM potassium phosphate pH 7.2 with HCl Protease inhibitor
Tris-HCl buffer	Base: • 0.5ml Tric HCL • 0.1ml EDTA • 0.3ml NaCl • 0.1ml NP-40 For HAM exposure supplemented with • 0.1 SDS Water to 10 ml

Table 2.6 - Antibodies

Antibody	Source/Product Number	Concentration
Western blot		
Caspase-1	AdipoGen (#AG-20B-0042-C100)	1:2000
NLRP3	AdipoGen (#AG-20B-0014-C100)	1:2000
ASC	Adipogen (#AG-25B-0006, AL177)	**WB**: 1:2000 **IF** 1:200
GAPDH	ThermoFisher(#MA5-15738)	1:2000
Cleaved IL-1β	Cell Signalling (#12426)	1:500
GSDMD	Abcam (#ab209845)	1:1000
Pro- IL-1β	Cell Signaling(#12426S)	1:750
EEA1	Invitrogen (#PA517228)	1:1000
Caveolin 1 (1:1,000)	ThermoFisher (Cat# MA3-600)	1:1000
Secondary antibodies • Anti-mouse • Anti-rabbit • Anti-goat	Invitrogen • A16066 • A16104 • A15999	1:5000
Microscopy		
Mouse Ly-6G/Ly-6C (GR-1) antibody	Biolegend (#108424)	0.25ug/ml
Anti-mouse CD11b antibody	Biolegend (#101212)	0.25ug/ml
Anti-TGN38/46 antibody (Clone 2F7.1)	Thermo Fisher(#MA3-063)	1:1000
Myc (Myc.A7), DyLight TM	Invitrogen (#MA1-21316-D550)	1:1500

2.8 Immunoblotting

Cell lysates were loaded onto SDS-PAGE gels (10–12%, based on target protein size, with 30 µL used for inflammasome-related experiments) and run at 80 volts for 30 minutes, followed by 100 volts for 80 minutes. Following electrophoresis, gels were removed and immersed in transfer buffer. Proteins were then transferred onto nitrocellulose membranes using the BioRad Trans-Blot® Turbo™ Transfer System for 30 minutes. After transfer, membranes were washed with TBS-Tween (0.5%) and then blocked in a 5% milk solution for 1 hour at room temperature with gentle shaking. Blocking buffer was removed, and membranes were incubated with the specified primary antibody (Table 2.6) overnight at 4°C with shaking. The next day, the primary antibody was removed, and membranes were washed for 5 minutes in TBS-Tween (0.05%) before incubating with the corresponding secondary antibody (Table 2.5) for 1 hour at room temperature with shaking. Membranes were washed three times in TBS-Tween and then exposed to Clarity Western ECL Blotting substrate for 2–3 minutes. Imaging was performed using the BioRad ChemiDoc Imaging system (**Table 2.3**).

2.9 Inflammasome reconstitution assay

HEK293T cells were cultured in DMEM supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin. Cells were maintained until confluency and underwent subculturing every 2–3 days. Transfections to reconstitute the NLRP3 inflammasome were performed using Lipofectamine 2000 in the presence of ATP (**Table 2.4**). Human plasmids included pBEN-NLRP3 N-Flag (200 ng), pBEN-Casp1 C-Myc (200 ng), and pBEN-ASC N-Myc (30 ng), generously provided by Prof. Thomas Miethke from University Medicine Mannheim, Germany (Waldhuber *et al.*, 2016). Mouse plasmids used included pLV-CMV-NLRP3-Hygro (200 ng), pcDNA3-N-Flag-caspase-1 (200 ng), and pcDNA3-N-Flag-ASC1 (30 ng), with the caspase-1 and ASC plasmids kindly provided by Bruce Beutler (Shi, Murray and Beutler, 2016).

2.10 ASC oligomerization

BMDMs were treated in a 6-well plate, washed with cold PBS, and lysed in 500 µL of 1% Triton X-100 buffer supplemented with protease inhibitors. The lysate was centrifuged at 6800 g for 20 minutes at 4°C to separate Triton X-100 soluble and insoluble fractions, after collecting a 50 µL aliquot for input control. The insoluble fraction (pellet) was cross-linked with 2 mM disuccinimidyl suberate (DSS) at room temperature with continuous agitation for 30 minutes. Cross-linked pellets containing ASC oligomers were then resuspended in 2x Laemmli buffer, boiled for 5 minutes at 95°C, and analysed on a 10% SDS-PAGE gel.

2.11 Endosome isolation

Endosomes were isolated from iBMDM cells cultured in 150 cm^2 tissue culture plates to an approximate density of 15 x 10^6 cells. The cells were treated with LPS and ATP, with or without FASN and palmitoylation inhibitors, as outlined in the previous section. Following the treatments, cells were scraped into ice-cold PBS and resuspended in 0.8 mL KPBS buffer (25 mM KCl, 100 mM potassium phosphate, pH 7.2), supplemented with a protease inhibitor cocktail. Cells were then disrupted by passing them 30 times through a 27G needle attached to a 1 mL syringe. The protein concentration of the post-nuclear supernatant was measured and normalised using a BCA assay. Samples were incubated overnight at 4°C with a rabbit anti-EEA1 polyclonal antibody (1:25 dilution) on an end-over-end rotator. The following day, 60 µL of Dynabeads coated with anti-Rabbit IgG were added to each sample and incubated overnight at 4°C with gentle rotation. The beads, which carried EEA1-positive endosomes, were then washed twice with 500 µL KPBS buffer containing the protease inhibitor cocktail using a magnetic stand. After the washes, the beads were resuspended in 100 µL of protein sample buffer, centrifuged, and loaded onto an SDS-PAGE gel.

2.12 Protein measurement by Pierce BCA assay

Protein concentration in cell lysates was determined using a BCA protein assay kit. Cell lysates (10–20 µL) and an equal volume of BCA protein standards were dispensed into a 96-well plate. A working reagent was prepared in a 50:1 ratio of Reagent A to Reagent B, with 200 µL added to the cell lysates in each well. The samples were incubated for 30 minutes at 37 °C, after which absorbance was measured at 560 nm using a plate reader. Protein standards were employed to generate a standard curve, from which the concentrations of unknown samples were extrapolated.

2.13 Acyl biotin exchange assay

To determine NLRP3 palmitoylation, a modified ABE assay was conducted on HEK293T cells overexpressing mouse NLRP3 constructs (Myc-NLRP3 WT, C671A, C835A, C898A) or on primary and immortalized BMDMs to evaluate the palmitoylation of endogenous NLRP3. Briefly, cells were transfected overnight or treated as indicated (Leishman, Aljadeed and Anand, 2024). Following treatment, lysates were collected and incubated overnight at 4 °C with 20 mM N-ethylmaleimide (NEM) in the presence of a protease inhibitor cocktail to block free thiol sites. Blocking was performed in Tris-HCl buffer containing 1 mM EDTA, 150 mM NaCl, and 1% NP-40. After protein precipitation, samples were resuspended in Tris-HCl with 5 mM EDTA, 150 mM NaCl, 1% NP-40, and either 1% SDS with 0.7 M hydroxylamine (HAM+) or without (HAM-) and incubated at room temperature for 1 hour before adding 20 mM Biotin-HPDP for an additional hour. Following protein precipitation, samples were exposed to fresh

20 mM Biotin-HPDP and incubated for another hour at room temperature with gentle rotation. Excess Biotin-HPDP was removed before incubating samples with pre-washed streptavidin agarose at 4 °C overnight. Samples were washed before resuspending them in 2x SDS-PAGE Laemmli buffer.

2.14 In vivo peritonitis model

An *in vivo* model of peritoneal inflammation was established using 8–12-week-old C57BL/6 mice. Mice were administered intraperitoneal injections of LPS (100 µg/kg) and either cerulenin (50 µg/kg), 2PB (100 µg/kg), or MCC950 (50 µg/kg), all dissolved in sesame oil. After 4 hours, mice received a secondary injection of either PBS or ATP for 15 minutes. The animals were then euthanised by carbon dioxide inhalation, and approximately 500 µL of blood was collected immediately. Serum samples were stored at -20 °C for subsequent cytokine level analysis using ELISA. To assess in vivo caspase-1 activity, peritoneal exudate cells were harvested in 3.0 mL of DMEM and resuspended in FACS buffer (1% BSA and 0.1% sodium azide in PBS). The cells were transferred to a 96-well plate, washed once with FACS buffer, and stained with FAM-FLICA caspase-1 (YVAD) for 1 hour at 37 °C to detect active caspase-1. Following staining, cells were washed twice and incubated with a mix of anti-mouse Ly-6G/Ly-6C (GR-1) antibody (0.25 µg/mL) and anti-mouse CD11b antibody (0.25 µg/mL) for 30 minutes at 4 °C. After incubation, the cells were washed twice, resuspended in FACS buffer, and analysed using a Cytoflex flow cytometer. Raw data were processed with FlowJo software. For the ABE assay, mice were injected with 2.0 mL of 3% thioglycollate medium, and peritoneal cells were harvested 4 days later in 3.0 mL of DMEM. The animals were then treated as described above, and peritoneal cells were collected for the ABE assay

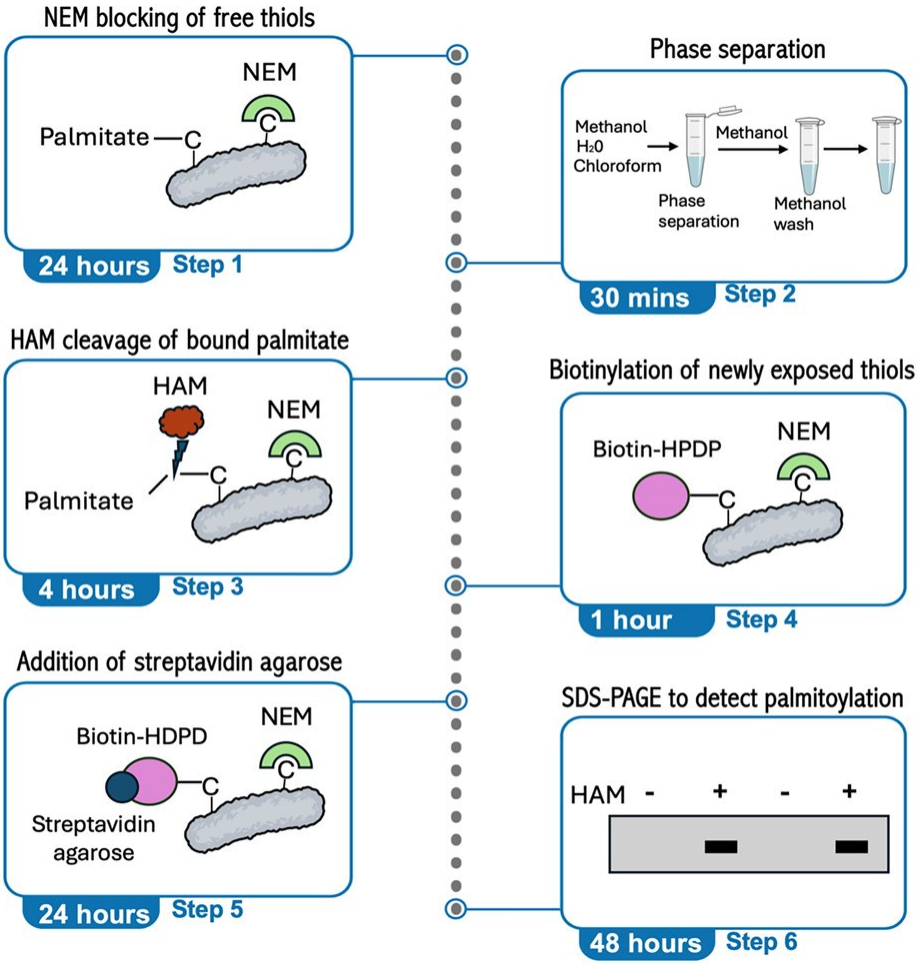


Figure 2.1: Acyl-Biotin Exchange protocol. Proteins were collected, and free cysteine sites were blocked with NEM. Following a series of separation steps, palmitoylated sites were cleaved using HAM, leaving unbound free cysteine sites. Biotin-conjugated HPDP was introduced for binding to these free cysteines, and extraction was performed using streptavidin agarose beads. Samples were subsequently analysed following the standard western blot protocol. Created with Biorender.com.

2.15 Indirect immunofluorescence

Indirect immunofluorescence microscopy and propidium iodide (PI) staining, primary and immortalized BMDMs, along with HEK293T cells, were plated on coverslips in a 12-well plate overnight for adhesion. Coverslips for HEK293T cells were coated with poly-lysine solution prior to cell plating. The following day, cells were treated as indicated in the figure legends. In some cases, cells were transfected overnight with Myc-NLRP3 WT and mutant constructs before treatment. At the conclusion of any treatment, cells were fixed in 4% paraformaldehyde (PFA) for 30 minutes at room temperature. Cells on coverslips were incubated with a PBS solution containing 50 mM glycine for 10 minutes at room temperature, followed by permeabilization with 0.1% Triton X-100 in PBS for 20 minutes on ice. After blocking with 1% BSA in PBS for 20 minutes at room temperature, cells were labelled with specific antibodies for 1 hour at room temperature. AlexaFluor-conjugated secondary antibodies were used for detection. PI staining was performed by incubating cells on coverslips with a 500 nM working solution for 5 minutes. Following the staining procedure, coverslips were mounted on slides with a drop of mounting medium containing DAPI to stain the nucleus. The slides were stored in the dark at room temperature overnight before imaging on a confocal microscope or a fluorescent microscope for PI images. The images were processed and analysed using ImageJ software.

2.16 Lactate dehydrogenase assay

Lactate dehydrogenase (LDH) activity was assessed using a colorimetric cytotoxicity assay. A total of 50 µl of supernatant was collected from samples obtained in prior experiments and transferred to a 96-well plate. Subsequently, 50 µl of reaction buffer was added to each well. The plate was incubated at room temperature for 30 minutes in the absence of light. Following incubation, 50 µl of stop solution was introduced to halt the reaction, and the absorbance was measured using a spectrophotometer at wavelengths of 490 nm and 680 nm. LDH values were expressed relative to the activated control, which was arbitrarily defined as 80% LDH release rather than total lysis.

2.17 Enzyme-linked immunosorbent assay

Cytokine secretion of IL-1β, IL-18, TNF-α, and IL-6 was measured using an ELISA. A total of 100 µl of capture antibody diluted in 1x coating buffer was added to each well of a 96-well ELISA plate and incubated overnight at 4 °C. The next day, the wells were washed with 250 µl of wash buffer (PBS-Tween 0.05%) and incubated with 200 µl of 1X EliSpot diluent for 1 hour at room temperature. After aspirating the diluent, 100 µl of samples and 100 µl of pre-prepared standards were added to the wells. The plate was incubated at room temperature for 2 hours. Following incubation, the wells were washed three times with wash buffer before

adding 100 µl of detection antibody for 1 hour. After another three washes with wash buffer, 100 µl of avidin-HRP antibody was added and incubated for 1 hour at room temperature. The wells were washed five times with wash buffer before adding the substrate TMB and incubating for about 15 minutes. The reaction was stopped with 2N H_2SO_4, and absorbance was measured at 450 nm and 570 nm. The 570 nm value was subtracted from the 450 nm reading, and cytokine concentrations of unknown samples were determined from the standard curve.

2.18 Liquid-Liquid extract of lipids

hBMDMs were differentiated and cultured overnight in a 6-well plate at a density of 3 × 10^6 cells per well, as previously described. The following day, cells were treated with LPS, ATP, or a combination of both to activate the canonical inflammasome. For the activation of the non-canonical inflammasome, cells were exposed to Pam3CSK4 or transfected with LPS. After the activation period, cells were lysed using methanol and placed on ice, with the supernatant collected for ELISA. Splash Lipidomix, generously provided by EMBL Germany, was added to each sample at a volume of 100 µl per condition. Following the addition of the internal standard, lipid extraction was performed via liquid-liquid phase separation using a methanol:water:cholofrom mixture (2:2:1 ratio). Methanol was evaporated using a speedvac for 1 hour, and samples reconstitute in 100 µL 90% IPA in 1.5mL tubes and stored at -80 until they were shipped on dry ice.

2.19 Liquid chromatography-mass spectrometry

Lipid extracts from canonical and non-canonical activated hBMDM were analysed at EMBL Germany. The analysis utilized a Kinetex C18 column (100 mm × 2.1 mm; 2.6 µm) and detection via Orbitrap Exploris 240. With a mobile phase comprising 10 mM ammonium formate and 0.1% formic acid in isopropanol (9:1) and 10 mM ammonium formate and 0.1% formic acid in water (6:4). The flow rate was maintained at 0.26 mL/min. The phases were mixed according to the specifications outlined in Table 2.7. Lipids were analysed in both positive and negative electrospray ionization (ESI) modes, with a spray voltage of 4.5 kV, a capillary temperature of 320 °C, and a probe heater temperature of 300 °C. The m/z ratios obtained were processed and analysed using MS-DIAL software. Figures for LC-MS were generated using Metaboanalyst software.

Table 2.7- LC-MS mobile phase

Time	% A	% B
0	80	20
0.5	80	20
3	50	50
10	30	70
18	3	97
22	3	97
22.1	80	20
25	80	20

2.20 Quantification and statistical analysis

GraphPad Prism 10.0 software was used for data analysis. Data are represented as mean ± SD or SEM (as indicated in the figure legends) and are representative of experiments conducted at least three times. The Shapiro-Wilk test was performed to assess the normality of the data. Based on the results of this test, either parametric or non-parametric tests were applied. The sample size for each experiment was determined based on power analysis, prior studies, and practical considerations, ensuring sufficient statistical power to detect a meaningful effect. A minimum of three independent experiments was performed; additional replicates were included for control groups. Statistical significance was determined using an unpaired Student's t-test or one-way ANOVA, with a significance threshold of $p < 0.05$. Lipidomic data relative abundance was acquired from MS-DIAL based on the m/z ratio. Abundance was first filtered using a variance filter in the interquartile range, and an abundance filter was applied based on the mean intensity value. Data scaling was performed using mean centring. PLS-DA models were generated, and corresponding VIP scores were calculated via metaboanalyst coding. Heatmaps were generated with auto-scaled features using the Ward clustering method.

Results

Chapter 3

Fatty acid synthesis is required for NLRP3 activation

Chapter 3. Fatty acid synthesis is required for NLRP3 activation

3.1 Introduction

Inflammasomes are multiprotein complexes that assemble in response to a variety of stimuli, including microbial infections, cellular stress, and environmental factors (Anand, Malireddi and Kanneganti, 2011; Shi *et al.*, 2014; Kelley *et al.*, 2019; Kumari *et al.*, 2020). These complexes typically consist of a sensor protein, which interacts with the adaptor protein ASC to recruit pro-caspase-1. The assembly of the inflammasome activates pro-caspase-1, leading to its cleavage into active caspase-1. Cleaved caspase-1 subsequently processes the inactive cytokines pro-IL-1β and pro-IL-18 into their active mature forms, which are key mediators of inflammation. Caspase-1 also cleaves GSDMD, whose N-terminal fragment translocates to the plasma membrane to form pores (He *et al.*, 2015; Liu *et al.*, 2016). These pores facilitate the release of cytokines and other intracellular contents, inducing a form of inflammatory cell death known as pyroptosis.

The NLRP3 inflammasome, one of the most studied inflammasomes, is activated through a two-step process upon detecting PAMPs or DAMPs (Martinon, Burns and Tschopp, 2002; Anand, Malireddi and Kanneganti, 2011; Broz and Dixit, 2016; Mangan *et al.*, 2018). Abnormal activation or dysregulation of the NLRP3 inflammasome has been linked to various autoimmune, infectious, and inflammatory diseases (Schroder and Tschopp, 2010; Jo *et al.*, 2015). Recent studies have also begun exploring the relationship between the NLRP3 inflammasome and immunometabolism—the interaction between immune and metabolic pathways—which has shown how metabolic changes can influence immune responses and vice versa.

The interplay between lipids and inflammasome activation has gained prominence as a pivotal focus within the field of immunometabolism. Metabolic pathways, including glycolysis, the TCA cycle, lipid metabolism, and purine metabolism, contribute to metabolic reprogramming that profoundly influences immune cell function (Choi *et al.*, 2011; Mills *et al.*, 2016; Kumar *et al.*, 2020; Olona, Leishman and Anand, 2022; Ortega *et al.*, 2023). These processes are essential for energy production and meeting the biosynthetic demands required to maintain effective immune responses, with different pathways being activated in response to a wide range of stimuli (Qiu *et al.*, 2023). Metabolic reprogramming operates at both micro and macro levels, with evidence from Traba et al. showing that refeeding following a fasting state confers resistance to NLRP3 inflammasome activation, likely through a reduced mitochondrial ROS

response in humans (Traba et al., 2015). On a micro scale, myriad lipids pathways can influence NLRP3 activation, to both activate or inhibit this complex

Research on NLRP3 activation shows that the sensor protein functions not only as a responder to external threats but also as a key regulator of cellular processes (Leemans, Cassel and Sutterwala, 2011). For instance, glucose-6-phosphate, the first intermediate in glucose metabolism, has been shown to enhance NLRP3 activation. This effect is thought to occur through the inhibition of hexokinase, a critical enzyme in glycolysis, in response to bacterial peptidoglycans (Wolf *et al.*, 2016). Further, regulation of NLRP3 via glucose-6-phosphate is hypothesised to involve the dissociation of hexokinase from the mitochondrial membrane channel VDAC (Baik *et al.*, 2023). This dissociation in turn allows for calcium influx from the ER to the mitochondria, and the generation of pores that allow for efflux of mtDNA in turn activating NLRP3 (Baik *et al.*, 2023). The regulation of NLRP3 by glucose-6-phosphate and hexokinase dissociation highlights how metabolic intermediates influence immune function, reinforcing the idea that NLRP3 is not merely a sensor of pathogens but also a key player in maintaining cellular homeostasis in response to metabolic changes.

Another example of metabolic regulation occurs in response to LPS signalling. When LPS is present, macrophages undergo a metabolic switch from the tricarboxylic acid (TCA) cycle in their resting state to glycolysis when activated. This shift supports rapid ATP production and biosynthetic demands (Sun *et al.*, 2022). The TCA cycle generates succinate, which acts as a signalling molecule in innate immunity (Harber *et al.*, 2020). Succinate production in response to LPS has been shown to enhance IL-1β production (Tannahill *et al.*, 2013). Furthermore, succinate dehydrogenase promotes increased mitochondrial oxidation of succinate, leading to heightened production of ROS, further amplifying inflammation (Mills *et al.*, 2016; Degen *et al.*, 2023). Both the succinate and glucose-6-phosphate pathways highlight how NLRP3 is regulated by metabolic pathways.

Lipids, acquired from dietary intake or from stored reservoirs in adipose tissue, are fundamental constituents of cells. Most notably, lipids make up plasma membranes, maintaining cellular homeostasis by regulating the barrier between endogenous and exogenous substances (Jackson, Walch and Verbavatz, 2016). Distinctly, they also can act as both pro-inflammatory and anti-inflammatory mediators (Andersen, 2022). Previous research implicated lipids such as cholesterol in NLRP3 inflammasome activation, due to the physical nature of cholesterol in its crystal form damaging the phagolysosome compartment (Duewell *et al.*, 2010a; Rajamäki *et al.*, 2010). Cholesterol, a critical sterol primarily obtained from exogenous dietary sources, plays a key role in stabilizing cell membranes and is essential for the function of transmembrane receptors and lipid rafts (Ikonen, 2008). Soluble cholesterol

has also been implicated in NLRP3 activation. One study demonstrated that cholesterol trafficking via the NPC1 endosome-lysosome pathways is crucial for NLRP3 inflammasome activation, with cholesterol accumulation in *Npc1*-deficent cells inhibiting NLRP3 oligomerisation (de la Roche *et al.*, 2018). This underscores the complex and multifaceted nature of lipid-dependent stimuli that can activate the inflammasome.

The role of lipid biosynthesis in NLRP3 activation has also been examined. Sterol biosynthetic pathways are activated when extracellular lipids are depleted, triggering SREBP (DeBose-Boyd and Ye, 2018). SREBPs remain in an inhibitor complex bound to transmembrane protein INSIG and SCAP, located in the endoplasmic reticulum (Matsuda *et al.*, 2001; Yang *et al.*, 2002; Shimano and Sato, 2017). Upon depletion of sterols, INSIG undergoes ubiquitination and a conformational change in SCAP, enabling SCAP-SREBP transport to the Golgi (Li *et al.*, 2013; Zhang *et al.*, 2013). Once at the Golgi, SCAP-SREBP is cleaved sequentially by S1P and S2P proteases, leaving the free N-terminal SREBP to translocate to the nucleus activating genes involved in lipid biosynthesis (Matsuda *et al.*, 2001). Of particular importance is SREBP1 that induce the expression of fatty acid synthase (FASN).

FASN, a dimeric enzyme with seven active sites, is crucial for the de novo synthesis of long-chain fatty acids (Wei *et al.*, 2016). The FASN enzyme extends the carbon backbone of short chain malonyl-CoA and acetyl-CoA, both products of other metabolic processes, through a series of cyclical reactions to generate palmitate (Wakil, Stoops and Joshi, 1983). When FASN is activated, its malonyl/acetyltransferase domain transfers acetyl and malonyl groups from their CoA chaperones. These lipids are then processed by ketoacyl synthase domains, which catalyse their condensation into acetoacetyl-ACP. ACP, a conserved protein, shuttles lipids between FASN domains, ensuring substrate tethering and lipid elongation (Nguyen *et al.*, 2014). Acetoacetyl-ACP is converted to β-hydroxybutyryl-ACP by the β-ketoacyl reductase site of FASN, followed by dehydration to form an unsaturated intermediate **(Fig 1.5)** (Wakil, Stoops and Joshi, 1983). It should be noted that although palmitate is produced in approximately 90% of cases, FASN has also been reported to synthesise minor quantities of myristic acid and stearic acid (Menendez et al., 2024). Furthermore, FASN is typically inactive under physiological conditions, though it becomes highly active under metabolic stress, such as in tumours, autoimmune diseases, and inflammatory disorders (Xiao et al., 2024). While this may seem counterintuitive, it is part of a more complex adaptive mechanism that helps the cell cope with stress. The increased FASN activity balances the energetic cost of fatty acid synthesis with the benefits of energy storage and the maintenance of cellular integrity, which is crucial for recovery (Rysman *et al.*, 2010; Menendez *et al.*, 2024). The heightened activity of FASN during stress or nutrient deprivation underscores its vital role in lipid synthesis, a key process for cellular adaptation to external stressors.

Palmitic acid and its salt form, palmitate, play numerous roles in cellular homeostasis, serving as a source of cellular energy, contributing to lipid biosynthesis, acting as a signalling molecule, and participating in the lipid modification of proteins, known as palmitoylation (Levental *et al.*, 2010; Carta *et al.*, 2015, 2017b; Dong *et al.*, 2020). Its role in energy generation is twofold. First, palmitate can be incorporated into triglycerides. These triglycerides are then stored in lipid droplets, serving as an energy reserve that can be mobilised when needed (Zadoorian, Du and Yang, 2023). Second, it undergoes β-oxidation, breaking down into acetyl-CoA to fuel the citric acid cycle and drive ATP production in mitochondria (Kakimoto *et al.*, 2021). Beyond its role in cellular energy, palmitate acts as a vital starting point for the synthesis of many long chain fatty acids. Examples include the phospholipids that make up cellular membranes (Carta *et al.*, 2015), and sphingolipids which act as signalling molecules and components of lipid rafts (Levental *et al.*, 2010). Palmitate, in particular, has diverse and essential functions and its availability is tightly regulated to maintain a balance between exogenous sources and biosynthesis by the FASN pathway. The balance in palmitate levels impacts membrane fluidity and integrity but may also relay lipotoxic effects when in excess (Collins *et al.*, 2010). This lipotoxicity is accredited to mitochondrial dysfunction and endoplasmic reticulum stress (Shi *et al.*, 2006; Xu *et al.*, 2015; Han and Kaufman, 2016).

Despite the well-characterized functions of FASN in cellular processes, much remains unknown about how its diverse roles are precisely regulated under various physiological and pathological conditions. The intricate balance between fatty acid biosynthetic pathway and exogenous lipid supply in maintaining membrane integrity and fluidity warrants further exploration to fully elucidate its contributions to cellular signalling and stress responses (Wei *et al.*, 2016; Lu *et al.*, 2019; Du *et al.*, 2024).The studies discussed above highlight a significant relationship between FASN activity and the signalling pathways involved in inflammasome priming. This represents a shift in the understanding of how molecular mechanisms influence inflammasome activation, emphasizing a more nuanced approach to the regulation of inflammasome assembly.

In this chapter, I investigate the interplay between FASN and the NLRP3 inflammasome, demonstrating that pharmacological inhibition of FASN modulates NLRP3 activation. Furthermore, I found that FASN promotes NLRP3 activation without affecting priming, and this is independent of upstream NLRP3 stimuli examined. My findings suggest that inhibition of FASN activity impairs pyroptosis due to the inability of caspase-1 to undergo autoproteolytic cleavage. Remarkably, these findings were unique to NLRP3, and other inflammasomes with distinct sensor proteins were not affected. Finally, I demonstrate that supplementation with palmitate can rescue NLRP3 inflammasome activity upon FASN inhibition, providing insight into the potential mechanisms mediating this relationship.

3.2 Results

3.2.1 Inhibition of FASN reduces caspase-1 activation, IL-1β secretion and pyroptosis in mouse bone marrow-derived macrophages

The relationship between FASN and NLRP3 remains largely unclear, though there is some connections are established. Carroll et al. (2018) showed that FASN is crucial for lipid raft formation, and FASN inhibition in BMDMs impaired NLRP3 priming, reducing pro-IL-1β expression (Carroll *et al.*, 2018). Blocking FASN's ketoacyl synthase activity, which prevents malonyl-ACP and acetyl-ACP from converting to acetoacetyl-ACP, lowered NLRP3 priming markers, including pro-IL-1β, TNF-α, and IL-10. They concluded that acetoacetyl-ACP influenced NLRP3 inflammasome priming, though its role in inflammasome activation was not explored.

To examine inflammasome activation, mouse primary BMDMs were stimulated with LPS, a well-established priming signal for the NLRP3 inflammasome, for 4 hours. This resulted in increased expression of the inflammasome components, particularly NLRP3 and pro-IL-1β. Following this, an NLRP3 agonist ATP, was added for 30 minutes to activate the NLRP3 inflammasome. ATP binding to the $P2X_7$ receptor induces potassium efflux and mtROS production (Hamilton *et al.*, 2022). Consequently, the NLRP3 inflammasome is activated, resulting in the cleavage of caspase-1 and induction of an inflammatory form of cell death, known as pyroptosis. As a result, the cells display characteristic features of pyroptosis, followed by plasma membrane rupture. The cell lysates were collected in lysis buffer containing protease inhibitors to evaluate the expression of inflammasome components by immunoblotting.

To examine the role of FASN on NLRP3 activation, cerulenin, a small molecule inhibitor of FASN, was used. Cerulenin, originally isolated from *Cephalosporium caerulens*, was initially recognized for its potent antifungal properties. However, its potent function as a FASN inhibitor has since been recognized (Fhu and Ali, 2020). Cerulenin irreversibly binds to the ketoacyl synthase domain of FASN, thereby inhibiting the conversion of malonyl-ACP and acetyl-ACP to acetoacetyl-ACP. Inhibition of FASN with cerulenin results in a reduction in downstream palmitate production and consequently fatty acid synthesis.

To assess the impact of FASN on inflammasome activation, cerulenin was administered 2 hours after the LPS priming signal, at which point NLRP3 was abundantly expressed. Subsequent activation of the NLRP3 inflammasome by ATP led to the cleavage of pro-caspase-1 into its active 20-kDa form. In the presence of cerulenin, a dose-dependent reduction in cleaved caspase-1 and cleaved GSDMD was observed (**Fig 3.1A**). However,

inflammasome constituents such as NLRP3, pro-caspase-1, and precursor form of GSDMD remained unaffected **(Fig 3.1A**). Additionally, no major changes in the housekeeping protein GAPDH were detected (**Fig 3.1A**).

Given the potential subjectivity of Western blot analysis, densitometric quantification of caspase-1 and GSDMD bands was performed to confirm that the observed reduction was statistically significant (**Fig 3.1B**). The densitometric analysis further validated that the reduction in caspase-1 observed in the presence of cerulenin was significant even at the lowest concentration tested. Intriguingly, supplementation of cerulenin exhibited a dose-dependent decrease in caspase-1 cleavage. To further validate these findings the experiment was repeated by employing alternative NLRP3 inflammasome triggers. PAM3CSK4, a synthetic triacylated lipopeptide and known TLR2/TLR1 ligand, was used to prime the NLRP3 inflammasome, while nigericin, an antibiotic that induces P2X7-independent potassium efflux leading to NLRP3 activation, was employed (Gurung *et al.*, 2015). In the presence of these ligands, exposure to cerulenin still results in a significant reduction in cleaved caspase-1 and mature IL-1β release (**Fig 3.1C**). These observations suggested that FASN regulates NLRP3 inflammasome activation independently of the upstream stimuli.

The effect on IL-1β secretion, a critical downstream consequence of NLRP3 activity, was examined in cells grown in the absence or presence of cerulenin. Supernatants were collected and cytokine levels for caspase-1 dependent IL-1β and inflammasome-independent TNF- α and IL-6 were measured (**Fig 3.2A-C**). IL-1β serves as a direct indicator of NLRP3 inflammasome activation and was dose-dependently inhibited in the presence of FASN inhibitors (**Fig 3.2A**). Concurrently, TNF-α and IL-6 levels were analysed, both of which remained unaffected, serving as controls for general cytokine release (**Fig 3.2B and 3.2C**). These results support the hypothesis that FASN plays a crucial role in regulating NLRP3 inflammasome activity. Further validation of these findings was achieved by analysing IL-1β secretion following stimulation with Pam3CSK4 and nigericin, confirming that the observed inhibition occurs across multiple NLRP3 stimuli (**Fig 3.2D**). While the findings were compelling, further validation using alternative pharmacological inhibitors of FASN was necessary to confirm that the observed inhibition was not an off-target effect of a cerulenin.

To further corroborate the previous findings, an alternative FASN inhibitor, C75, was employed. C75, a cerulenin derivative, targets the same ketoacyl synthase site but with greater stability due to its modified structure (Flavin *et al.*, 2010). Similar to cerulenin, macrophages exposed to C75 after LPS priming exhibited comparable levels of NLRP3, ASC, pro-casapse-1 and GSDMD but a dose-dependent decrease in the expression of the p20 subunit of caspase-1 and N-terminal GSDMD (**Fig 3.3A**). Additionally, no changes were detected in the

housekeeping protein GAPDH. To quantify the decrease observed by immunoblotting, densitometric analysis on blots from different experiments was performed. This confirmed a significant inhibition of both cleaved caspase-1 and the GSDMD N-terminal subunit upon exposure to FASN inhibitors (**Fig 3.3B**). To further validate the role of the FASN pathway in inflammasome activation, the cytokine profile of C75 exposed macrophages was examined. Dose-dependent inhibition of FASN reduced IL-1β release, while TNF-α and IL-6 levels remained unaffected. (**Fig 3.3C**).

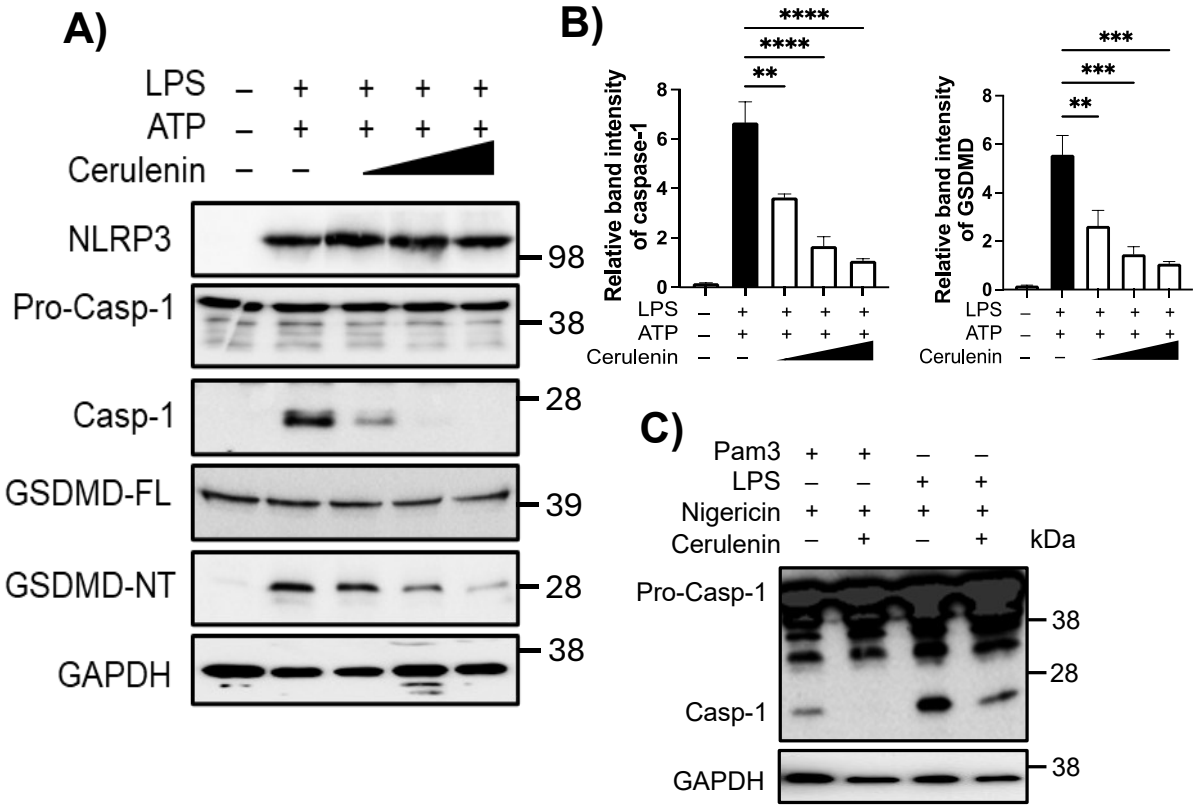


Figure 3:1: Effect of FASN on NLRP3 inflammasome activation. **A)** Immunoblots from LPS-primed (500ng/ml; 3.5h) BMDMs exposed to cerulenin (6.25, 12.5, and 25 μM) before exposure to ATP (5mM; 1h). **B)** Relative band intensity when exposed to increased concentrations of cerulenin. **C)** Immunoblots from BMDMs exposed to the TLR2 agonist Pam3CKS4 (500 ng/mL; 3.5 h) and nigericin (20 μM; 1 h) or LPS and nigericin in the absence or presence of cerulenin.; $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$, by one-way ANOVA.

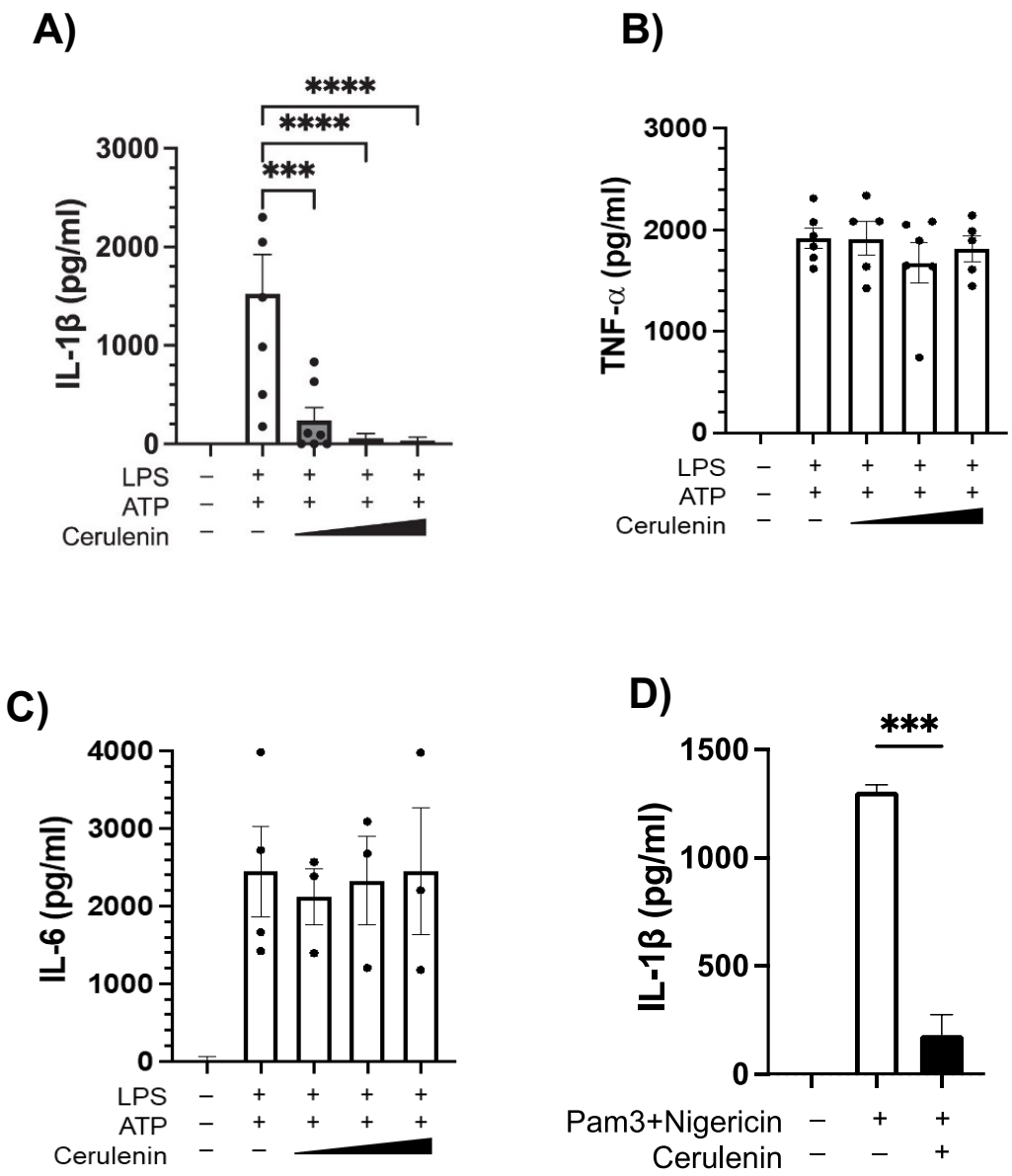


Figure 3.2: Functional FASN is essential for the secretion of cytokines associated with NLRP3 activation. A) IL-1β release from LPS-primed BMDMs exposed to cerulenin (6.25, 12.5, and 25 μM) before exposure to ATP. **B)** TNF- α from cells treated as before**. C)** IL-6 from cells treated as before. **D)** IL-1β release from BMDMs exposed to the TLR2 agonist Pam3CKS4 (500 ng/mL; 3.5 h) and nigericin (20 μM; 1 h) or LPS and nigericin in the absence or presence of cerulenin. Data shown are mean ± SD, and experiments shown are representative of at least three. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$, by one-way ANOVA, or Student's t test

3.2.2 Inhibition of NLPR3 via FASN prevent permeabilization of cell membranes

Caspase-1 activation cleaves GSDMD, forming plasma membrane pores and inducing pyroptosis (Liu et al., 2016a). To assess this, PI staining was used to identify NLRP3 inflammasome-activated cells undergoing cell death. Fluorescent microscopy showed that FASN inhibition with cerulenin or C75 prior to ATP stimulation prevented pyroptosis-related pore formation (**Fig. 3.4A, 3.4B**), supporting the hypothesis that FASN is essential for NLRP3 inflammasome activation and GSDMD cleavage.

These results were corroborated by measuring LDH release, an indicator of plasma membrane rupture during cell death (**Fig. 3.4C**). In this case, cell lysis was arbitrarily defined as 80%. While this approach allows for standardised comparisons across conditions, it does not represent absolute cell death. Future experiments could be improved by including a total lysis control to allow for more accurate quantification. To determine whether FASN's role was stimulus-specific, alternative activation signals, such as Pam3 (TLR2 agonist) and nigericin (potassium efflux inducer), were used. FASN inhibition significantly reduced propidium iodide staining across these stimuli, demonstrating that cell membrane permeabilization is not limited to a single TLR or NLRP3 pathway (**Fig. 3.4D, 3.4E**). These findings indicate that NLRP3 induced pyroptosis depends on FASN activity.

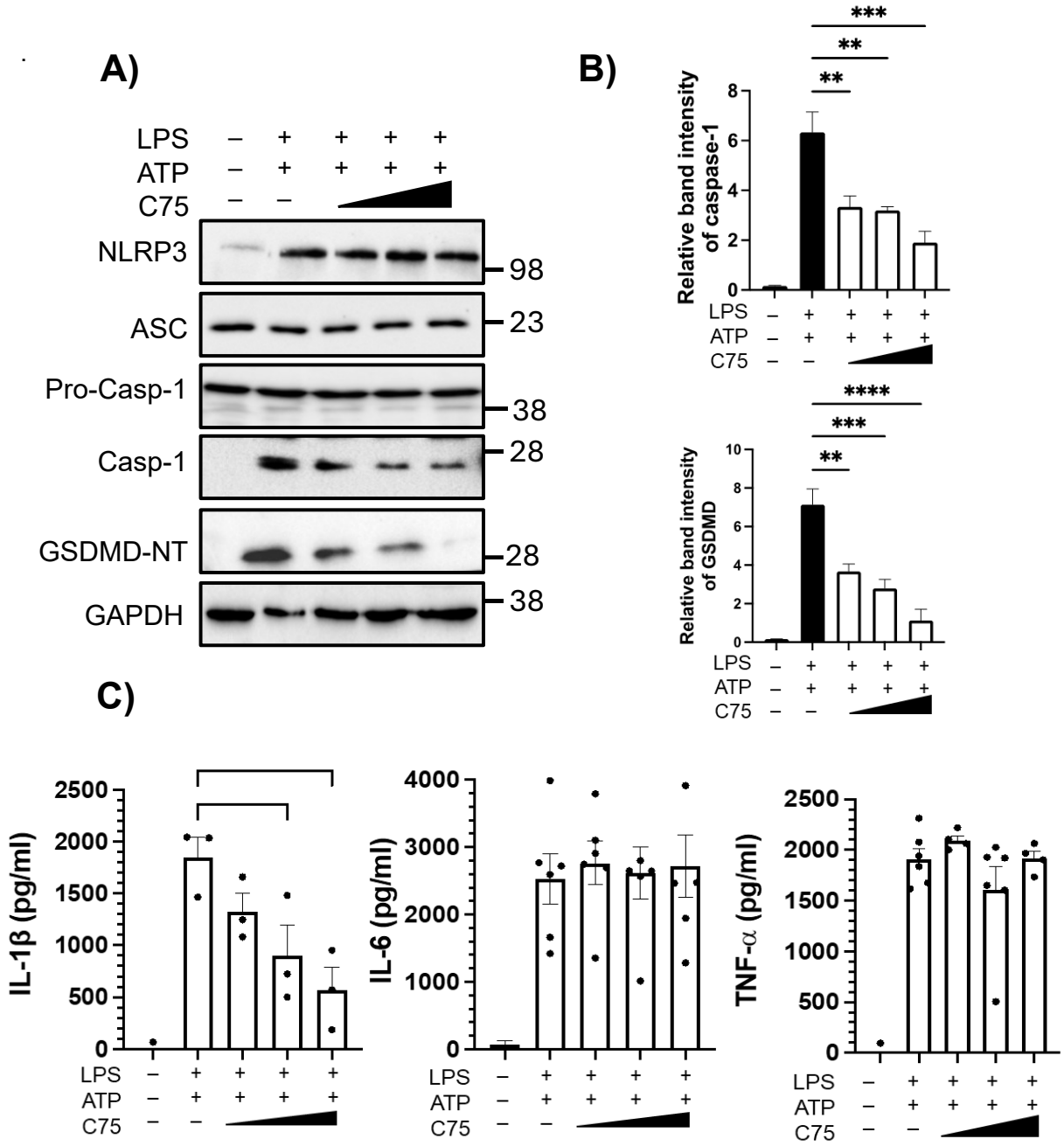


Figure 3.3: FASN inhibition at the ketoacyl synthase site with distinct inhibitors influences NLRP3. A) Immunoblots from LPS-primed (500ng/ml; 3.5h) BMDMs exposed to the FASN inhibitor C75 at increasing concentrations (12.5, 25, and 50 μM) prior to stimulation with ATP (5mM; 1h). **B)** Relative band intensity of cleaved caspase-1 and GSDMD. **C)** IL-1β, IL-6 and TNF-α released from cells treated with C75. Data shown are mean ± SD, and the experiments shown are representative of at least three independent experiments**.** Scale bars, $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$, by one-way ANOVA, or Student's t test

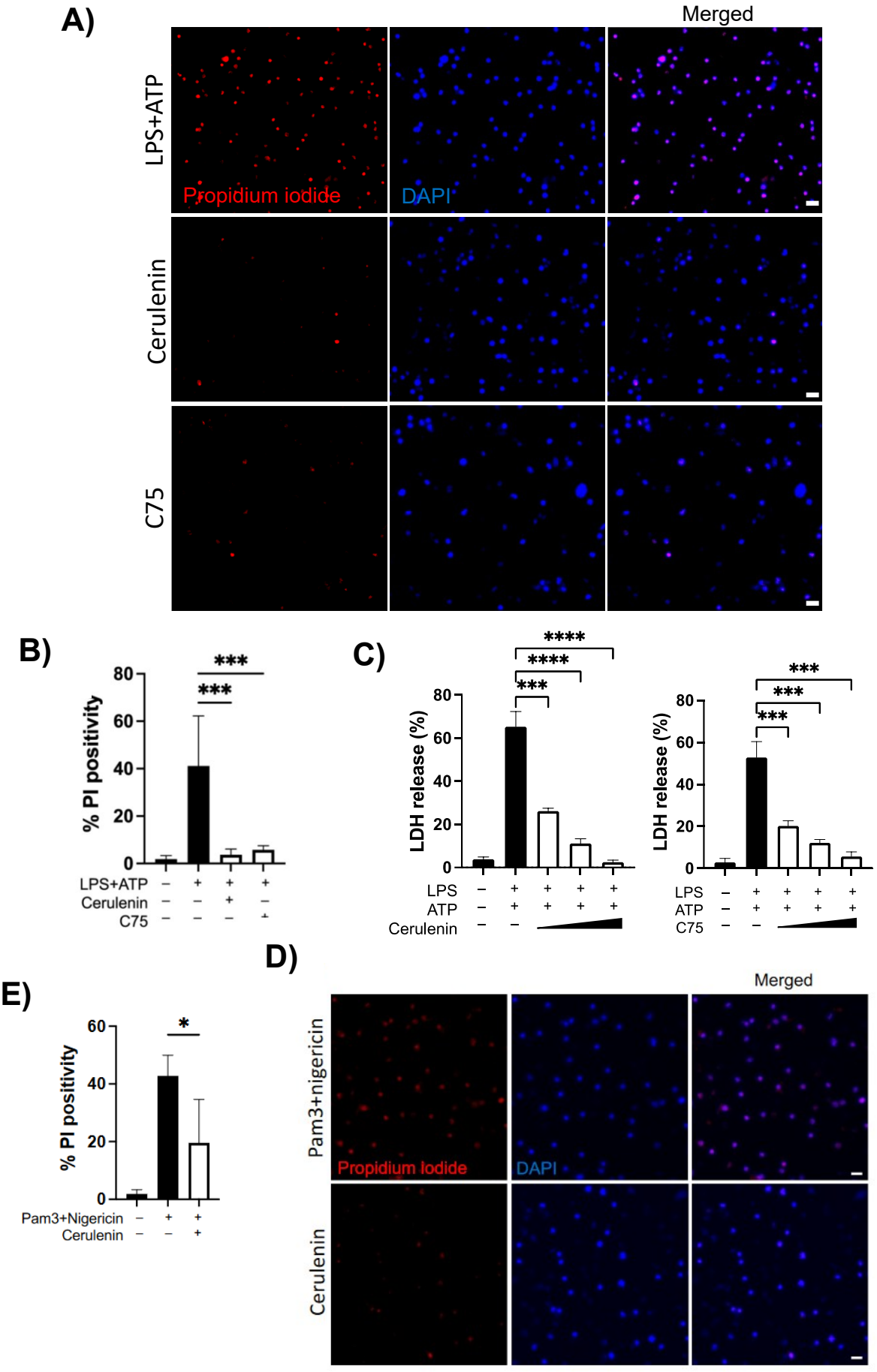

A)
Merged
LPS+ATP
Cerulenin
C75
Propidium iodide
DAPI
B)
% PI positivity
LPS+ATP
Cerulenin
C75
C)
LDH release (%)
LPS
ATP
Cerulenin
C75
D)
Merged
Pam3+nigericin
Cerulenin
Propidium Iodide
DAPI
E)
% PI positivity
Pam3+Nigericin
Cerulenin

Figure 3.4: FASN induces membrane permeabilization by NLRP3. A) Fluorescent images of BMDMs treated with either cerulenin or C75, stained with propidium iodide (PI). **B)** Percentage of PI-positive cells from the experiment in A. **C)** LDH release from BMDMs treated with either cerulenin or C75. **D)** BMDMs exposed to the TLR2 agonist Pam3CSK4 and nigericin (20 μM), in the absence or presence of cerulenin. Fluorescent images of BMDMs treated as described, stained with propidium iodide and DAPI. **E)** Percentage of PI-positive cells from the experiment described in D. Data shown are mean ± SD, and experiments shown are representative of at least three independent experiments. Scale bars, 15 μm. *, p<0.05; **, $p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$, by one-way ANOVA, or Student's t tests.

3.2.3 FASN promotion of inflammasome assembly is not specific to the ketoacyl domain

Given FASN's six catalytic domains, the roles of domains other than the previously examined ketoacyl synthase were investigated in NLRP3 inflammasome activation using orlistat and GSK2194069 (Hardwicke *et al.*, 2014). Orlistat and GSK2194069 act downstream of the ketoacyl synthase site, but remain critical for palmitate production. Orlistat, a drug used in obesity management, functions as a reversible inhibitor of the thioesterase domain of FASN, which is essential for converting butyryl-ACP to palmitate, the final stage of the FASN pathway (Kridel *et al.*, 2004). On the other hand, GSK2194069 inhibits the conversion of acetoacetyl-ACP to β-hydroxybutyryl-ACP, directly downstream of the ketoacyl synthase site. Upon exposure to these inhibitors, a significant reduction in the expression of cleaved caspase-1 was observed (**Fig 3.5A**), along with a decrease in secreted IL-1β, while TNF-α levels remained unaffected (**Fig 3.5B and 3.5C**). At the same time, there was a significant reduction in PI positive cells investigated, suggesting loss of membrane permeabilization when orlistat and GSK2194069 were present (**Fig 3.5D**) These findings further corroborate FASN is essential for NLRP3 inflammasome activation and that the role of FASN in NLRP3 activation is not restricted to any one particular domain.

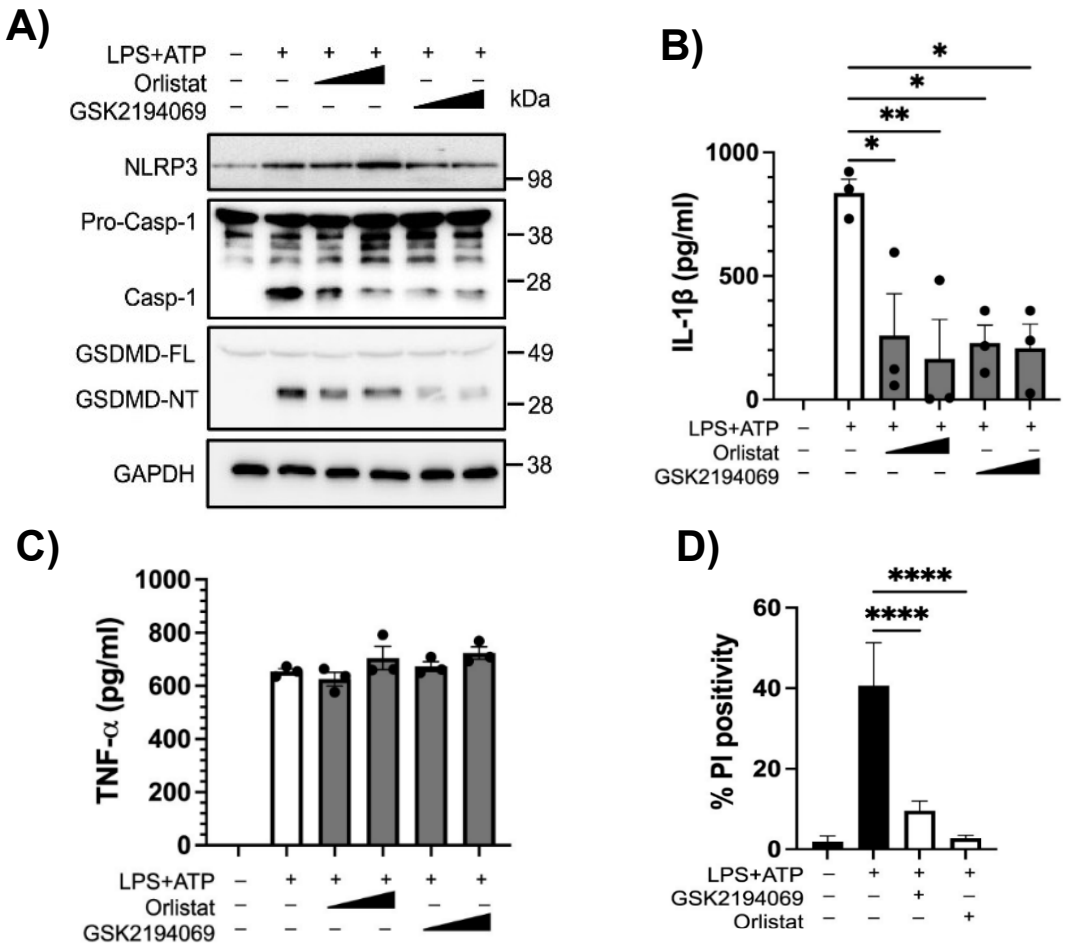


Figure 3.5: Acetyl-CoA:ACP transacylase and Ketoacyl Reductase domains are essential for FASN's role in promoting inflammasome activation. **A)** Immunoblots from LPS-primed BMDMs exposed to either GSK2194069 (1, 2 μM) or orlistat (100, 200 μM). **(B, C, D)** IL-1β and TNF-α release, and percentage of PI positivity from cells treated as in **(A),** Data shown are mean ± SD, and the experiments shown are representative of at least three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$, by one-way ANOVA.

3.2.4 Palmitoylation is required for inflammasome activation

FASN catalyses the synthesis of palmitate, a precursor for fatty acids like stearic and oleic acids, and a key player in mammalian cell metabolism (Carta et al., 2017). Palmitate can also undergo palmitoylation, covalently attaching to cysteine, serine, or threonine residues of target proteins, affecting their stability, localisation, and function. We hypothesised that inflammasome inhibition might be linked to palmitoylation of its components by palmitate.

To test this, we blocked protein palmitoylation by exposing cells to a competitive analogue of palmitate, 2-bromopalmitate (2-BP). 2-BP blocks the incorporation of palmitate onto proteins, and whilst its exact mechanism is unknown, it is suspected to inhibit palmitoyl acyl-transferase enzymes that carry out protein palmitoylation protein palmitoylation (Davda *et al.*, 2013). Mouse macrophages were primed with LPS, followed by 2-BP treatment two hours before inflammasome activation, with cerulenin as a FASN inhibitor control. NLRP3 activation was assessed by detecting cleaved caspase-1, GSDMD N-terminal, and IL-1β. 2-BP significantly reduced these cleaved fragments compared to control cells, as confirmed by densitometric analysis (Fig. 3.6A, 3.6B).

Cell viability assays revealed a decrease in LDH release with FASN or palmitoylation inhibitors, indicating reduced cell death (Fig. 3.6C). IL-1β secretion was significantly reduced under these conditions, while TNF-α levels remained unaffected (Fig. 3.6D). These findings highlight a functional link between FASN, palmitoylation, and inflammasome activation, suggesting that FASN's role in inflammasome regulation may be mediated by protein palmitoylation.

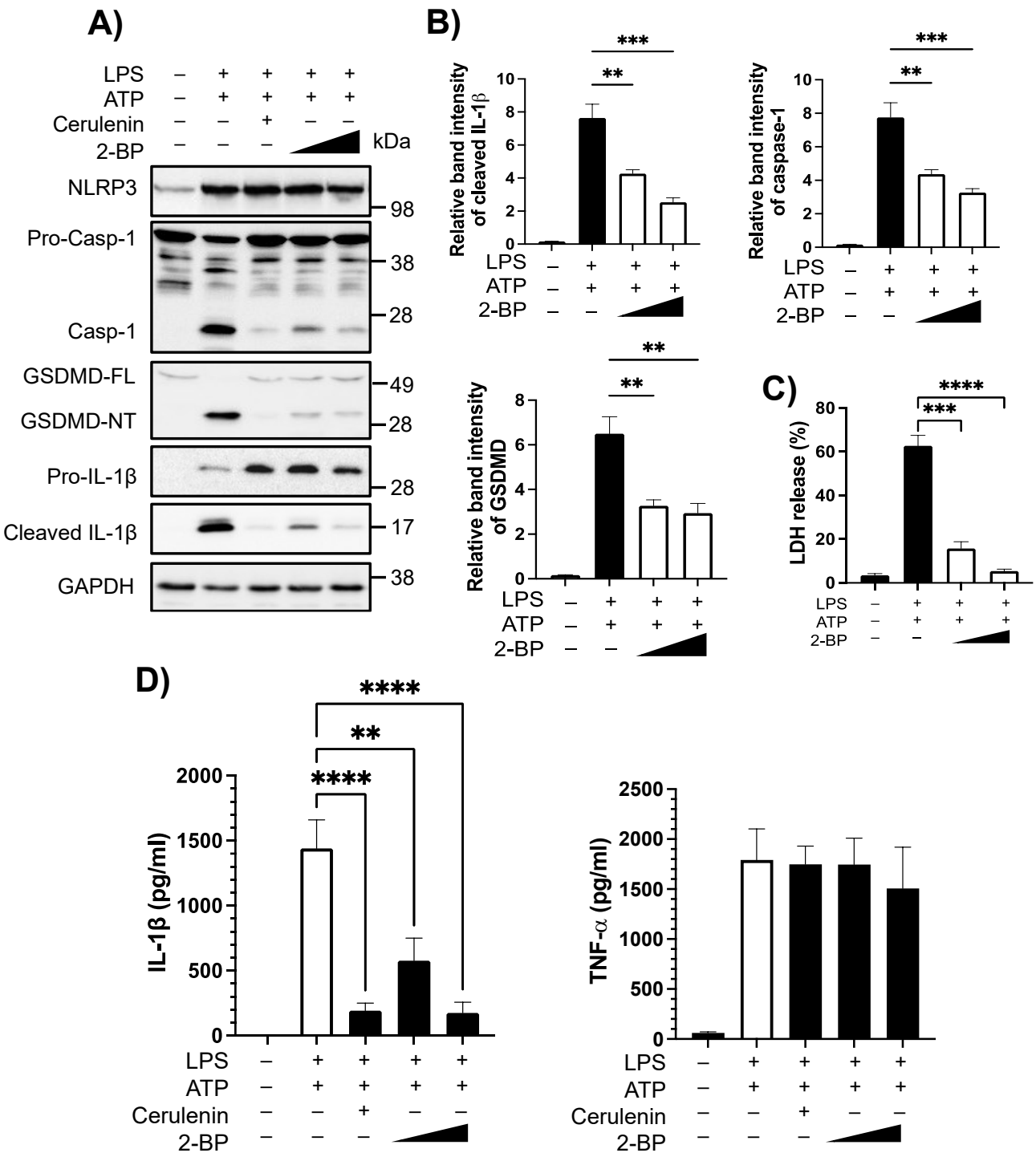


Figure 3.6: Palmitoylation is essential to NLRP3 inflammasome activation. A) Immunoblots from BMDMs exposed to LPS then either cerulenin (12.5 μM) or the palmitoylation inhibitor 2-BP (50 and 100 μM), followed by stimulation with the NLRP3 agonist ATP. **B)** Relative band intensity of cleaved caspase-1, GSDMD-NT, and cleaved IL-1β. **C)** LDH release from BMDMs treated with 2-BP. **D)** IL-1β and TNF-α release from BMDMs treated as described above. Data shown are mean ± SD, and the experiments shown are representative of at least three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$, by one-way ANOVA.

3.2.5 Effect of overnight inhibition of FASN and palmitoylation on cell viability and protein expression.

FASN depletion has been reported to exhibit cytotoxic effects, which underpins its potential as an anti-cancer therapeutic strategy (Fhu and Ali, 2020). To confirm the activity of our inhibitors and further ensure that they are not cytotoxic, BMDMs were exposed to FASN inhibitors either overnight or acutely for 2 hours. Exposure to FASN inhibitors overnight resulted in a cytotoxic profile, whereas no cytotoxicity was observed under light microscopy following the 2-hour treatment. Cell death was not detected under these conditions without NLRP3 activation (**Fig. 3.7A**). Thus, inhibition of FASN is cytotoxic with prolonged exposure but not with acute treatment, avoiding confounding cytotoxic effects. Interestingly, while 2-BP is an irreversible inhibitor, it did not cause any cytotoxicity during short-term treatment, suggesting that palmitoylation may not be critical for short-term cell viability (**Fig. 3.7B**). Western blot analysis confirmed these findings: macrophages treated with cerulenin overnight showed reduced expression of all proteins, including GAPDH, which could be indicative of cell death however further confirmation is required. In contrast, overnight 2-BP exposure did not alter protein expression (**Fig. 3.7C**). These results demonstrate that while prolonged suppression of functional FASN is cytotoxic, short-term treatment has no terminal effect.

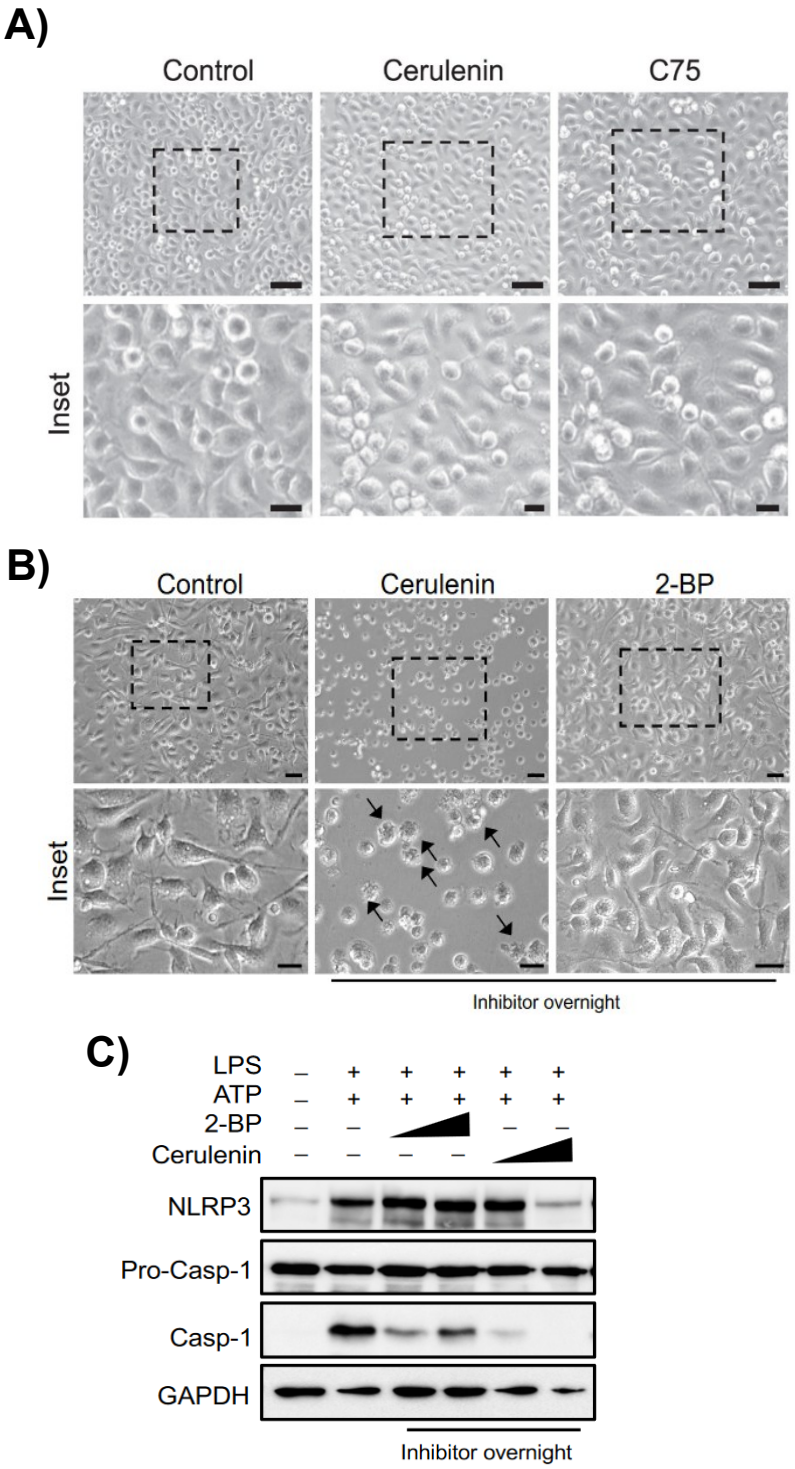


Figure 3.7: Effect of overnight inhibition of FASN and palmitoylation on cell viability and protein expression. A) Light microscopy images of LPS-primed BMDMs treated with either cerulenin or C75. **B)** Light microscopy images of BMDMs treated overnight with the FASN inhibitor cerulenin or the palmitoylation inhibitor 2-BP. Insets show magnified views of the highlighted areas. **C)** Immunoblots of cell lysates from BMDMs treated as described in B. Lysates were probed with the indicated antibodies, with GAPDH used as a loading control. Data are representative of at least three independent experiments. Scale bars, 25 µm (top images) and 10 µm (bottom inset images).

3.2.6 Palmitoylation is specific to the NLRP3 inflammasome

Previous results suggested the regulation of the NLRP3 inflammasome by FASN/palmitoylation axis. To next examine whether this regulation extends across other inflammasomes, AIM2 and NLRC4 inflammasomes were activated in the presence of inhibitors for FASN and palmitoylation. The AIM2 inflammasome is triggered in response to cytosolic double stranded DNA and acts as a defence mechanism for viral infection (Kumari *et al.*, 2020a). Not requiring the priming and activation signals typical of NLRP3, AIM2 is constitutively expressed at a basal level during homeostasis but can be upregulated in response to interferon signalling (Brunette *et al.*, 2012). To trigger the AIM2 inflammasome, primary BMDM were transfected with double-stranded DNA in the presence of either cerulenin or 2-BP. Notably, it was found that neither FASN nor palmitoylation impacted the AIM2 inflammasome (**Fig 3.8A**). Consequently, the expression of cleaved caspase-1 remained comparable to that of control cells. Similarly, the levels of secreted IL-1β and TNF-α, were found to be consistent with those in control cells that were not exposed to any inhibitors (**Fig 3.8B, 3.8C**).

A parallel experiment was conducted for the NLRC4 inflammasome. One of the most well defined inflammasome adaptor proteins, NLRC4 responds to a wide array of NLR apoptosis inhibitor proteins, which in turn are able to bind to an array of bacteria type 3 secretion system structures (Sundaram and Kanneganti, 2021). To induce NLRC4, BMDMs were infected with *Salmonella typhimurium* for 2 hours in the absence of antibiotics. After 6 hours, cell lysates and supernatants were collected for analysis. No significant changes in the levels of cleaved caspase-1 were observed in the presence of either FASN or palmitoylation inhibitors, even at the highest doses, suggesting that FASN and the subsequent inhibition of palmitoylation do not impact the NLRC4 inflammasome (**Fig 3.8D**). This conclusion was corroborated by examining the levels of secreted IL-1β and TNF-α, both of which displayed no alterations in the presence of FASN and palmitoylation inhibitors (**Fig 3.8E**). Collectively, these findings indicate that only the NLRP3 inflammasome is affected by the FASN and palmitoylation pathway.

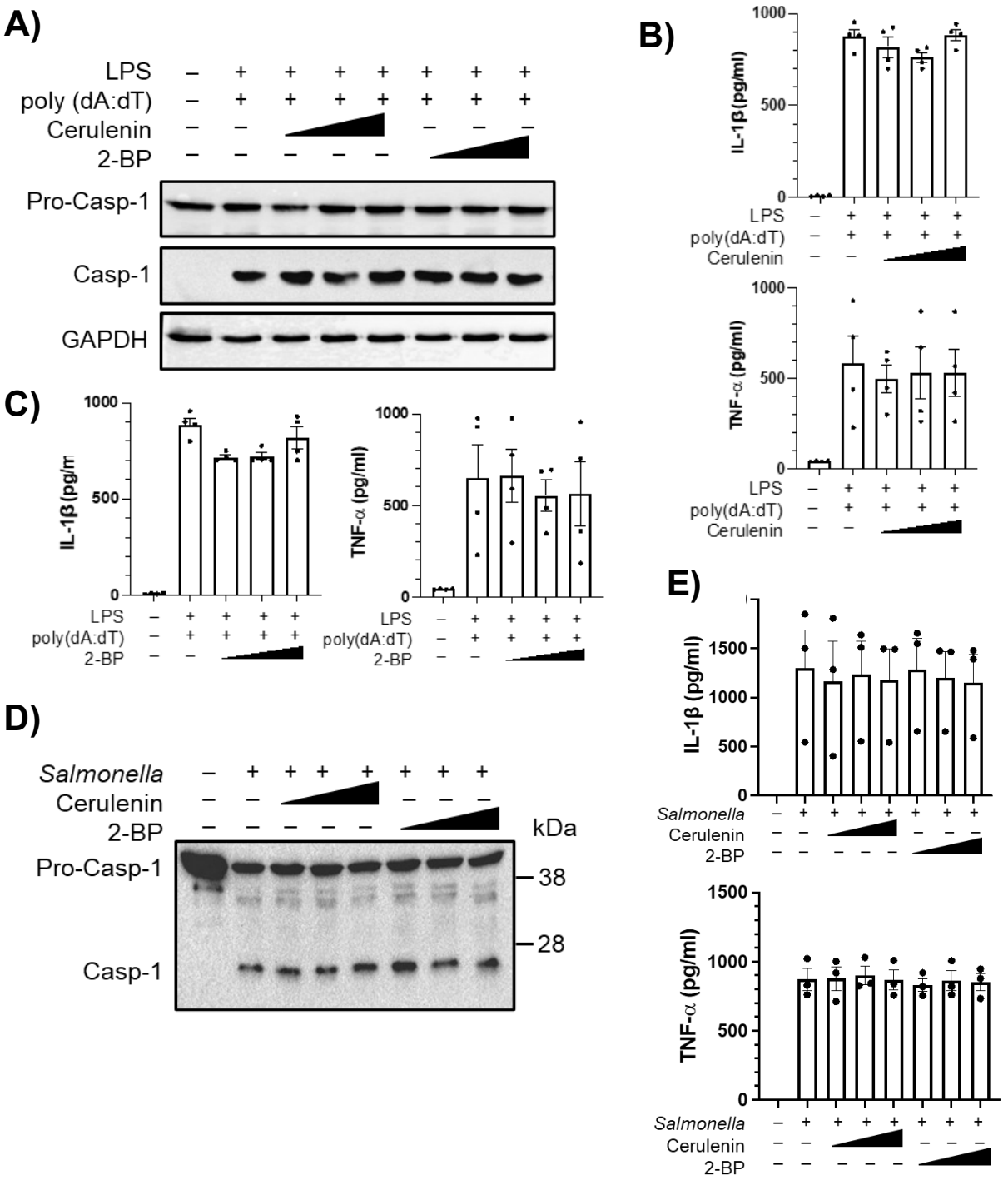


Figure 3.8: Regulation of the Inflammasome via FASN is specific to NLRP3. A) immunoblots from LPS-primed BMDMs exposed either to cerulenin (6.25, 12.5, and 25 μM) or to the palmitoylation inhibitor 2-BP (25, 50, and 100 μM) in cells transfected with AIM2 agonist poly(dA:dT) (1ug) overnight. **B,C)** IL-1β and TNF-α released from cells treated as in A. **D)** Immunoblot for caspase-1 and GAPDH of BMDMs infected with *S. typhimurium* at an MOI of 2 for ~4 h. During the last 2 hours, cells were exposed to increasing concentrations of cerulenin or 2-BP. **E)** TNF-a and IL-1β release measured by ELISA. Data shown are mean ± SD, and the experiments shown are representative of at least three independent experiments.

3.2.7 Exogenous palmitate rescues caspase-1 activation in the NLRP3 inflammasome

Given that 2-BP inhibited the NLRP3 inflammasome, it was speculated that the addition of exogenous palmitate would rescue caspase-1 activation and compensate for the lack of palmitate resulting from FASN inhibition. In support of this hypothesis, the addition of BSA-conjugated palmitate to cells effectively countered the inhibitory effect of 2-BP, resulting in rescued caspase-1 activation in macrophages exposed to NLRP3 stimuli. Notably, palmitate alone had no effect; control cells exposed to the NLRP3 stimulus and supplemented with BSA-palmitate exhibited no changes in caspase-1 processing compared to cells exposed solely to the NLRP3 stimulus (**Fig 3.9A**). Collectively, these findings suggest that palmitate production is critical for the activation of the NLRP3 inflammasome.

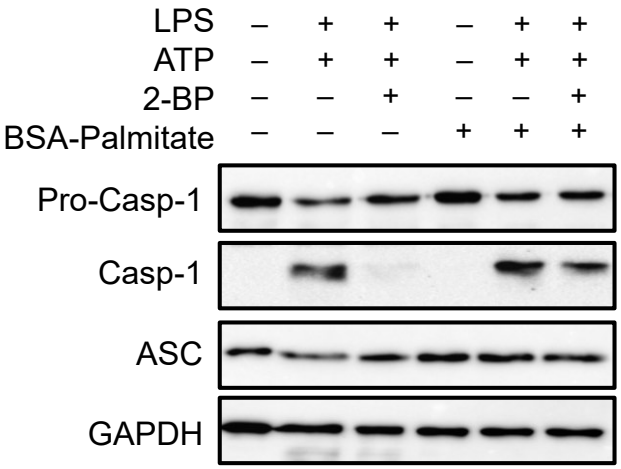


Figure 3.9: Supplementation of BSA palmitate rescues NLRP3 inflammasome activation. BMDMs treated with LPS priming signal were additionally exposed to BSA-conjugated palmitate (100 μM) overnight in the indicated samples in the presence or absence of 2-BP (100 μM). Cell lysates were immunoblotted with the indicated antibodies. Data shown is representative of at least three independent experiments.

3.3 Discussion

This chapter explored the connection between the NLRP3 inflammasome and the lipid biosynthesis enzyme FASN. This relationship was first identified through inhibition of the ketoacyl transferase site of FASN using the small molecule inhibitors cerulenin and C75. These inhibitors were observed to reduce the caspase-1 p20 subunit and N-terminal GSDMD levels, while simultaneously inhibiting IL-1β secretion. Fluorescence microscopy further supported these findings, revealing decreased pyroptosis in NLRP3-stimulated cells treated with FASN inhibitors, as evidenced by reduced uptake of the membrane-impermeable nuclear stain propidium iodide. Subsequent investigation using additional FASN inhibitors, including orlistat and GSK, confirmed FASN's involvement in NLRP3 inflammasome activity, regardless of the catalytic domain targeted. Similarly, the palmitate analogue 2-BP, an inhibitor of palmitoylation, produced comparable inhibitory effects, reinforcing the interplay between FASN and the NLRP3 inflammasome. Notably, this relationship appeared specific to NLRP3, as neither the AIM2 nor NLRC4 inflammasomes were affected by FASN or palmitoylation inhibition. Finally, exogenous palmitate rescued the inhibition of caspase-1 cleavage caused by FASN blockade, providing further evidence of palmitate's role in inflammasome activation. While the precise mechanism by which FASN influences NLRP3 activation remains unclear, these findings underscore the importance of FASN activity and palmitate production in fully activating the NLRP3 inflammasome. This research also highlights a broader role for lipid biosynthesis in inflammasome modulation, supporting evidence that NLRP3 regulation is influenced by diverse metabolites (Luheshi *et al.*, 2012; Anand, 2020; Niu *et al.*, 2021).

High-fat diet and inflammation are major contributors to metabolic diseases such as insulin resistance and type 2 diabetes, with IL-1β as a key inflammatory cytokine in their pathogenesis (Maedler *et al.*, 2002; Hotamisligil, 2006; Jager *et al.*, 2007). Elevated lipid availability is a strong predictor of type 2 diabetes, partly due to its ability to promote proinflammatory cytokine production through TLR activation (Roden, 2006; Nguyen *et al.*, 2007; Dong *et al.*, 2020). Increasing evidence suggests that the inflammasome plays a central role in these processes. For example, Wen et al. demonstrated that prolonged palmitate exposure (24 hours) stimulates NLRP3 inflammasome activation through the cleavage of pro-IL-1β, a mechanism linked to ROS production and suppression of AMPK. AMPK, a key regulator of fatty acid metabolism, inhibits ROS generation by suppressing NADPH oxidase activity (Wang *et al.*, 2010; Wen *et al.*, 2011b). Their findings support the broader link between lipid metabolism and inflammasome activation. However, their work focused exclusively on the long-term effect of saturated fatty acids. By contrast, the research in this chapter explored the acute relationship between palmitate and inflammasome activation, specifically investigating the role of FASN

activity in modulating this response. These findings aim to provide novel insights into the early inflammatory responses that underpin metabolic diseases.

Another study by Wei et al. demonstrated that deficiency of FASN could protect against diet-induced insulin resistant and inflammation. Their findings revealed that FASN regulates macrophage function through Rho GTPase trafficking, a mechanism essential for cell adhesion, migration, and activation (Wei *et al.*, 2016). FASN-deficient cells displayed altered membrane composition and reduced cholesterol retention in the plasma membrane, disrupting lipid raft formation and lowering the expression of diet-induced inflammatory markers. Notably, Wei et al. found that only endogenous cholesterol, and not palmitate, could restore a pro-inflammatory environment in FASN-deficient macrophages (Wei *et al.*, 2016). While there are parallels between their study and mine, important differences may explain the contrasting results. For instance, contrary to their study, exogenous palmitate rescued inflammasome activation in my experiments. One possible reason is that Wei et al. primarily used c-Jun N-terminal kinase (JNK) as their inflammation marker, which may overlook contributions from direct inflammasome components critical to cytokine processing (Han *et al.*, 2013). Moreover, they did not investigate the effects of palmitate in the presence of a priming signal, such as LPS, relying instead on pretreatment with palmitate. Since palmitate alone cannot prime the inflammasome, this difference highlights the potential influence of priming on exogenous palmitate pathways (Wen *et al.*, 2011b). Despite these differences, both their data and the research in this chapter underscore the pivotal role of FASN in regulating inflammatory responses. My findings provide additional insights into how exogenous palmitate influences inflammasome activity, particularly in the context of priming, advancing our understanding of FASN's role in inflammation.

Carroll et al. (2018) expanded on the concept of FASN involvement in inflammasome priming by demonstrating that FASN activity is essential for producing inflammatory cytokines, including TNF-α, IL-6, and IL-1β, in response to TLR4 activation (Carroll et al., 2018). They proposed that the intermediary lipid acetoacetyl-ACP, derived directly from malonyl-ACP and acetyl-ACP via the ketoacyl site, plays a pivotal role in influencing the cholesterol biosynthesis pathway. Specifically, their findings indicated that acetoacetyl-ACP is crucial for the colocalization of TLR4 and gangliosides within lipid rafts, a process necessary for initiating the signalling cascade required for TLR signalling and thus inflammasome priming. Moreover, they showed that inhibiting acetoacetyl-ACP disrupted lipid raft formation and subsequent cytokine production (Carroll et al., 2018). While this study provided valuable insights into the connection between FASN and TLR4-mediated priming, it focused narrowly on LPS-mediated pathways and did not explore a direct link between FASN and inflammasome activation. In this chapter, I provide evidence for a distinct relationship between FASN and NLRP3, one that appears to

function independently of FASN-derived intermediary metabolites like acetoacetyl-ACP and their role in cholesterol biosynthesis. My findings instead highlight the downstream role of palmitate, demonstrating that this interaction occurs after inflammasome priming and independently of TLR4 signalling. This distinction advances the understanding of FASN's involvement in inflammasome activation, suggesting alternative mechanisms that bypass the previously established function of FASN in TLR4 signalling.

Cholesterol biosynthesis and transport are well-established as regulators of NLRP3 (Li *et al.*, 2013; de la Roche *et al.*, 2018; Guo *et al.*, 2018). Growing evidence highlights a significant relationship between FASN and cholesterol, with both pathways drawing from the same acetyl-CoA pool, creating a complementary relationship. This interconnection is particularly notable in activated macrophages, where both pathways are upregulated (Langston, Shibata and Horng, 2017). Recent findings by Trub et al. (2022) demonstrate a direct interaction between the two pathways, specifically involving the enzyme HMG-CoA reductase (HMGCR) (Trub *et al.*, 2022a). HMGCR is the first rate-limiting enzyme in the cholesterol biosynthesis pathway, catalysing the conversion of HMG-CoA to mevalonate, a precursor for cholesterol synthesis (Singh *et al.*, 2013). Interestingly, Trub et al. suggests a more direct relationship: inhibition of HMGCR induces a specific post-translational modification of FASN (Trub *et al.*, 2022a). This modification, observed only in a small subset of FASN proteins near HMGCR, occurs in proximity to the endoplasmic reticulum and Golgi, and was associated with HMG-CoA accumulation (Trub *et al.*, 2022b). These findings suggest a highly localised regulatory mechanism between FASN and cholesterol biosynthesis, reinforcing the importance of metabolic compartmentalisation in activated macrophages. This interplay may represent a novel axis of regulation that integrates fatty acid and cholesterol synthesis to fine-tune inflammatory responses, particularly through its impact on NLRP3 activation.

To elucidate how palmitate regulates the inflammasome, the palmitate analogue 2-BP was utilised. 2BP is a synthetic analogue of palmitate, which acts as broad spectrum inhibitor of protein palmitoylation (Linder and Deschenes, 2007b; Davda *et al.*, 2013). In this study, 2-BP was shown to inhibit caspase-1 activation, GSDMD-D cleavage, and the production of mature IL-1β in mouse macrophages, demonstrating that palmitoylation is essential for inflammasome activation. These findings align with recent research indicating that acyltransferases, the enzymes responsible for palmitoylation, are stabilised in macrophages exposed to LPS (Zheng *et al.*, 2023). Furthermore, a recent study revealed that elevated ROS levels increase the expression of two acyltransferase enzymes, which were implicated in an inflammasome-activating ROS feedback loop mediated by GSDMD (Du *et al.*, 2024). Together with the results presented here, this suggests that during the priming phase of inflammasome activation,

palmitoylation enzymes are also equipped to facilitate inflammasome activation. The exact mechanism by which palmitoylation regulates inflammasome activity remains unclear.

The findings presented in this chapter add to the growing body of research showing that endogenous lipids can both suppress and activate inflammasome activity, underscoring a finely regulated balance (Iyer *et al.*, 2013; Guo *et al.*, 2018; Karasawa *et al.*, 2018). However, the intracellular mechanisms by which lipids influence inflammasome function remain poorly understood, particularly given that established stress signals can activate NLRP3 without a clearly defined mode of interaction. Emerging insights into lipid-mediated disorders and inflammasome activity reveal a direct link between NLRP3 activation and metabolic lipid disorders associated with chronic conditions such as diabetes, obesity, and hypertension (Duran-Salgado, 2014; Krishnan *et al.*, 2016; Hou *et al.*, 2018). As the prevalence of metabolic disorders continues to rise in western societies, research into the interplay between lipids and inflammation is increasingly critical for the development of novel therapeutics.

While pharmacological inhibition is useful, it has potential limitations, such as non-specific off-target effects or compensation by other enzymes with similar functions, which could confound results. Prolonged exposure to inhibitors may also induce cellular stress responses, influencing the outcome, which is of particular concern for inflammasome study due to the sensitive nature of NLRP3 to cellular stress (Bauernfeind *et al.*, 2011). To address these limitations, FASN knockout models can be used to provide a more specific and direct assessment of FASN's role in inflammasome activation, eliminating off-target effects and compensatory mechanisms (Zhang *et al.*, 2020; Cuyàs *et al.*, 2024). This approach would complement pharmacological inhibition and help validate the observed findings. However, due to the essential role of FASN, it would need to be implemented in an inducible form, such as a doxycycline-regulated system, to allow controlled switching on or off of the inhibition.

In conclusion, within this chapter, I suggest a previously unknown relationship between the inflammasome and the lipid biosynthetic pathway of FASN. This interplay was specific to NLRP3 inflammasome, independent of the mode by which it is activated but contingent upon protein palmitoylation. These findings reveal a novel regulatory axis in which changes in lipid metabolism modulate NLRP3-driven inflammatory responses. These findings underscore the dynamic nature of inflammasomes in their capacity to adapt to cellular metabolic states.

Results

Chapter 4

Palmitoylation is required for the localisation of NLRP3 to the dispersed *trans* Golgi network

Chapter 4: Palmitoylation is required for the localization of NLRP3 to the dispersed *trans* Golgi network

4.1 Introduction

PTMs have been shown to either enhance or inhibit NLRP3 inflammasome activity, depending on the specific site and the cellular context in which it occurs (Ramazi and Zahiri, 2021; Xia *et al.*, 2023). Phosphorylation of NLRP3 at Ser194 in mouse (Ser198 in humans) via JNK1 directly facilitates deubiquitination of NLRP3, a crucial step for activation of the NLRP3 inflammasome (Song *et al.*, 2017). Other sites such as Ser3 in mouse (Ser5 in humans) negatively regulate activation of NLRP3 via phosphorylation of the pyrin domain by PKA and PKD, hindering oligomerization (Stutz *et al.*, 2017). Additionally, recruitment of other components to the NLRP3 inflammasome complex is also affected by phosphorylation. For example, the NEK7 protein, which is believed to facilitate the formation of the NLRP3 cage structure, recruits and binds NLRP3 only when the sensor protein is phosphorylated at the Ser803 residue (Sharif *et al.*, 2019; Niu *et al.*, 2021). Serine is not the only target of phosphorylation enzymes with NLRP3 Tyr859 in mice (Tyr861 in humans) being phosphorylated by Bruton's tyrosine kinase, which negatively regulates NLRP3 assembly (Ito *et al.*, 2015; Bittner *et al.*, 2021). Overall, phosphorylation at diverse NLRP3 residues regulates NLRP3 inflammasome activation.

Distinct from phosphorylation, ubiquitination appears to exclusively suppress inflammasome activation. Ubiquitination involves the attachment of ubiquitin, a small regulatory protein that binds to lysine residues through a cascade of ubiquitin-activating, conjugating, and ligating enzymes (Liu *et al.*, 2020; Sun and Zhang, 2022). It has long been established that deubiquitination is essential for NLRP3 activation and that NLRP3 undergoes multiple rounds of deubiquitination depending on the stimulus. For example, LPS induces deubiquitinase activity, which is in turn inhibited by mtROS scavengers (Juliana *et al.*, 2012). In contrast, these scavengers fail to affect deubiquitination induced by ATP. This suggests that multiple rounds of deubiquitination allow for the fine-tuning of NLRP3 activation in response to different stimuli (Juliana *et al.*, 2012). For example, NLRP3 is deubiquitinated by BRCA1/2- containing complex subunit 3 (BRCC3) in response to LPS, which in turn allows for subsequent activation signal to induce inflammasome assembly (Juliana *et al.*, 2012; Lopez-Castejon *et al.*, 2013; Py *et al.*, 2013). Research has shown that the deubiquitinase enzymes USP7 and USP47 regulate inflammasome activation in macrophages (Palazón-Riquelme *et al.*, 2018). Their activity increases in response to inflammasome activators and their inhibition blocked inflammasome formation (Lopez-Castejon *et al.*, 2013; Palazón-Riquelme *et al.*, 2018). Interestingly, this study observed that USP7 and USP47 did not influence cytosolic LPS

stimulation of the caspase-11 inflammasome suggesting distinct ubiquitination pathways between canonical and non-canonical inflammasome (Palazón-Riquelme *et al.*, 2018). Another study demonstrated that NLRP3 is ubiquitinated by the E3 ubiquitin ligase GP78, which inhibits NLRP3 oligomerization and subcellular translocation (Xu *et al.*, 2022). Notably, this ubiquitination is facilitated by the endoplasmic reticulum membrane-bound protein INSIG (Xu *et al.*, 2022). INSIG is also involved in sterol regulation via the SCAP-SREBP pathway, which governs the expression of genes involved in cholesterol and lipid biosynthesis (Brown and Goldstein, 1997). When NLRP3 is ubiquitinated in this manner, it is recognised and degraded by E3 ligases, such as TRIM31, potentially explaining the low basal levels of NLRP3 in the absence of full inflammasome activation (Song *et al.*, 2016). Taken together, these results demonstrate that NLRP3 inflammasome activation is tightly regulated, ensuring that its activation is finely controlled through a balance of PTMs.

Whilst phosphorylation and ubiquitination are the widely studied examples of PTMs associated with NLRP3, other PTMs have also been investigated. In the last few years, SUMOlation has been identified to regulate NLRP3 inflammasome (Barry *et al.*, 2018; Galván-Peña *et al.*, 2019). SUMOylation is a reversible PTM process in which a small ubiquitin-like modifier (SUMO) binds to lysine residues within proteins and can be removed via SUMO-specific peptidase-mediated deSUMOylation (Hay, 2013). The relationship between NLRP3 and SUMO as a PTM is dichotomous. For instance, deSUMOlyation of NLRP3 Lys689, via SUMO-specific proteases, leads to increased ASC oligomerization and caspase-1 activation (Barry *et al.*, 2018). Similarly, SUMOylation of Lys204 also facilitates ASC oligomerization and the subsequent inflammasome assembly via SUMO1 (Shao *et al.*, 2020).

Recent studies describing the structure of active NLRP3 and the association of NLRP3 to membrane structures have underscored the critical role of inflammasome localisation in ensuring its stable and effective activation (Shankar S. Iyer *et al.*, 2013a; Chen and Chen, 2018a; Andreeva *et al.*, 2021). The precise spatial organisation facilitates interactions amongst inflammasome constituents, necessary for inflammasome assembly and function, highlighting its complexity as a tightly regulated molecular system (Akbal *et al.*, 2022). Upon receiving the priming signal, NLRP3 is induced and distributed in the cytoplasm, ready to detect danger signals (de la Roche *et al.*, 2018). In this dispersed form, it exists as a monomer or dimer, and has been reported to co-localise with the mitochondria, ER and Golgi (Zhou *et al.*, 2011; Chen and Chen, 2018a; Que *et al.*, 2024). It should be noted that NLRP3 has been associated with multiple cellular organelles, suggesting there may be a broad range of NLRP3 localisation sites or that the localisation may change depending on the upstream stimuli.

Mitochondrial localisation was originally hypothesised due to the large number of signals produced at this compartment that can induce NLRP3 activation, and it has been shown to be an essential step for NLRP3 assembly (Subramanian *et al.*, 2013; Abais *et al.*, 2015; Baik *et al.*, 2023). The association of NLRP3 to the mitochondria is mediated by the mitochondrial phospholipid cardiolipin (Shankar S. Iyer *et al.*, 2013b). This recruitment is thought to occur in the priming phase of NLRP3 stimulation in which cardiolipin translocated from the inner to outer mitochondrial membrane due to mitochondrial stress, and is dependent on ROS production (Elliott *et al.*, 2018). The relationship between NLRP3 and mitochondria is reinforced by stimuli that induce mitochondrial dysfunction. These have been suggested to include mtROS-mediated NLRP3-NEK7 interaction, oxidation of newly synthesized mtDNA, and mtDNA release into the cytosol, all driving inflammasome assembly activation (Nakahira *et al.*, 2011; Shi *et al.*, 2016; L. Zhang *et al.*, 2018). Consequently, autophagic removal of damaged mitochondria negatively regulates this process, potentially mediated by NF-κB–induced p62 accumulation (Nakahira *et al.*, 2011; Zhong *et al.*, 2018b). However, it has been proposed that mitochondrial damage may serve both as a downstream consequence and a contributor to NLRP3 inflammasome activation (Zhong *et al.*, 2018b).

Alternatively, studies have suggested that NLRP3 is located in the cytosol and associated with the endoplasmic reticulum (ER) under resting conditions (Zhou *et al.*, 2011). Upon activation both NLRP3 and ASC relocate to the ER and mitochondrial outer membranes that are in close proximity, also known as MAMs or mitochondrial-associated ER membranes (Zhou *et al.*, 2011). This positioning is thought to facilitate NLRP3's detection of cellular stress, predominantly one that is associated with unfolded protein response in the ER (Lerner *et al.*, 2012). Notably, cholesterol homeostasis—primarily regulated in the ER—plays a critical role in inflammasome activity by creating an intracellular environment conducive to NLRP3 inflammasome activation (de la Roche *et al.*, 2018). When NPC1, a cholesterol transporter, is inhibited, NLRP3 fails to recruit to mitochondria and ER membranes to activate the inflammasome (de la Roche et al., 2018). This has been proposed to rely on the integrity and fluidity of ER membranes, which are disrupted under conditions of cholesterol depletion.

NLRP3 inflammasome has been very recently suggested to be localised to the TGN compartment, shown by NLRP3 positivity in TGN38-positive vesicles (Chen and Chen, 2018a). Upon TLR ligation, the TGN disassembles into vesicles termed the dispersed *trans* Golgi network (dTGN) enriched with the negatively-charged lipid PI4P. Interaction between NLRP3 and PI4P facilitate NLRP3 assembly with dTGN acting as a scaffold for NLRP3 aggregation into multiple puncta (Chen and Chen, 2018). The mechanism by which NLRP3 recruits to PI4P-rich TGN38-positive vesicles is not fully understood but may involve NLRP3 phosphorylation (Zhang *et al.*, 2017a). However, the interaction between NLRP3 and the

negatively charged PI4P is mediated via electrostatic attraction to the KKKK motif in the polybasic region of NLRP3. This underscores a critical regulatory mechanism that is highly dependent on both spatial organisation and molecular context within the cell (Chen and Chen, 2018). Interestingly, subsequent studies have shown that PI4P is also enriched within endosomes and that the TGN38-positive vesicles that were originally believed to be *trans* Golgi derived are endosomal in nature (Lee *et al.*, 2023; Zhang. *et al.*, 2023) . The idea of NLRP3 endosomal association is further supported by studies demonstrating caspase-1 cleavage of the endosomal tethering protein EEA1 (Baroja-Mazo *et al.*, 2019).

It has been recently shown that inactive NLRP3 exists in a monomeric form and that when it activates, it initially forms a double-ring cage structure with its PYD domain inside the cage and LRRs forming the cage structure (Andreeva *et al.*, 2021; Yu *et al.*, 2024). The formation of a cage-like structure is believed to be a prerequisite for active NLRP3 inflammasome assembly. Interestingly, this structure has so far only been observed on Golgi membranes, further supporting a critical role for this organelle in inflammasome activation (Andreeva *et al.*, 2021; Yu *et al.*, 2024). While the precise contributions of the ER, mitochondria, and Golgi to NLRP3 localisation and activation remain under debate, emerging research has added further complexity.

As was shown in the previous chapter, FASN can influence NLRP3 inflammasome activation. The major product of FASN is the 16 carbon length lipid palmitate, which makes up the building block of all long chain fatty acids, and additionally has a distinct role in palmitoylation (Swinnen *et al.*, 2003; Collins *et al.*, 2010). Palmitoylation is the process whereby palmitate is covalently bound to proteins at primarily cystine, but also serine, threonine, or lysine residues (Linder and Deschenes, 2007). Palmitoylation has been reported to facilitate the translocation and localisation of proteins to their sites of activity (Linder and Deschenes, 2007b; Guan and Fierke, 2011). However, whether palmitoylation modifies NLRP3 and directs its translocation to TGN38-positive structures remains unknown.

The aim of this chapter is to build upon previous findings that established a strong connection between fatty acid synthesis and NLRP3 activation. While it has been demonstrated that fatty acid synthesis is critical for inflammasome activation, the precise mechanisms underlying its regulatory influence remain unclear. In this study, various approaches are explored to further elucidate the role of FASN and its involvement in lipid regulation and NLRP3 activation.

4.2 Results

4.2.1 *In silico* confirmation analysis of NLRP3 palmitoylation on inflammasome components

So far within this thesis, it has been demonstrated that FASN and palmitate production is essential for NLRP3 inflammasome activation. However, the precise mechanism by which this interplay occurs remains unclear. One of the key functions of palmitate is in palmitoylation, whereby palmitate is attached to proteins aiding protein stability, function, and localisation. To elucidate whether any components of the inflammasome undergo palmitoylation, *in silico* approaches were employed to predict putative palmitoylation sites within the amino acid sequences of mouse NLRP3, ASC, and caspase-1. This was accomplished using GPS-Palm program, which uses deep learning based on gene-proximity algorithms and large scale experimental data to identify sites of palmitoylation (Ning *et al.*, 2021). It was determined that no palmitoylation sites were present within the ASC and caspase-1 proteins, while multiple sites were identified within the NLRP3 sequence. Specifically, six conserved sites were detected (**Fig 4.1A**). Notably, five of these sites are located within the leucine-rich repeat (LRR) domain of NLRP3, while one site is situated in the linker region between the NACHT and LRR domains (**Fig 4.1A**). Out of the 5 sites within the LRR region, three of the cysteine sites were conserved across five different species tested (**Fig 4.1B**). Panel 4C shows a few of the putative cysteine residues on the NLRP3 AlphaFold structure (**Fig 4.1C**).

4.2.2 Palmitoylation regulates inflammasome activity upstream of GSDMD.

Recent findings by Balasubramanian et al. and others established that palmitoylation of GSDMD at the Cys191/192 residues (human/mouse) is essential for pyroptosis (Du *et al.*, 2023; Zheng *et al.*, 2023; Balasubramanian *et al.*, 2024). To unambiguously validate that observations made are independent of downstream pyroptosis, immortalised WT and *Gsdmd*$^{-/-}$ were exposed to NLRP3 stimuli LPS and ATP in the presence or absence of FASN and palmitoylation inhibitors. The levels of cleaved IL-1β were assessed to determine inflammasome activity (**Fig 4.2A**). The results indicated a significant increase in IL-1β levels in *Gsdmd*$^{-/-}$ cells compared to WT controls; however, this elevation was abolished upon inhibition of FASN and palmitoylation. Overall, these findings suggest that fatty acid biosynthesis and palmitoylation plays a critical role in regulating distinct steps of inflammasome signalling. Moreover, they indicate that the observed inhibition of caspase-1, upstream of pyroptosis, is a unique mechanism to regulate inflammasome activity.

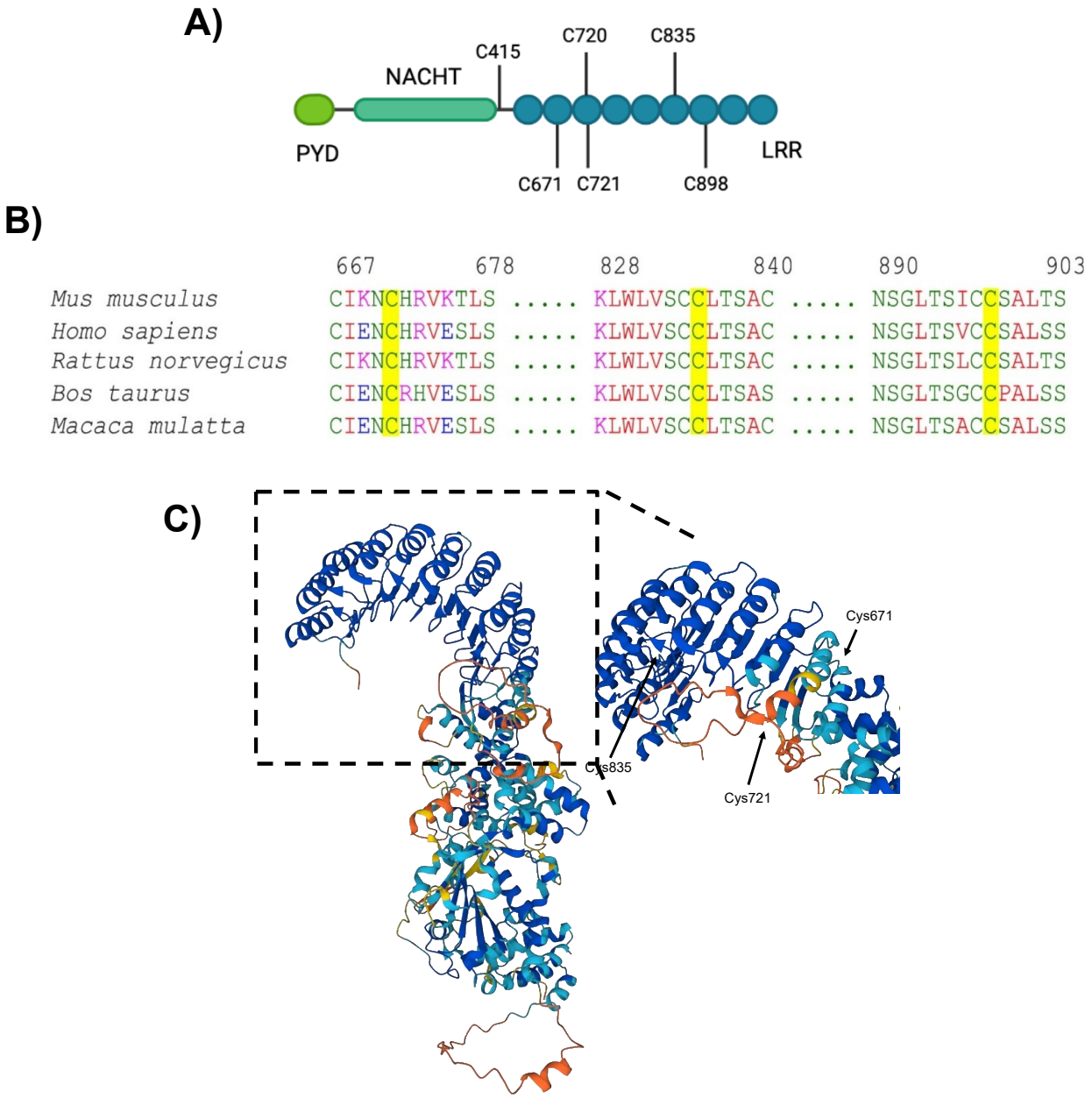


Figure 4.1 : *In silico* confirmation of NLRP3 palmitoylation. **A)** Schematic depicting the palmitoylation sites identified in the mouse NLRP3 amino acid sequence by GPS-Palm. **B)** Alignment of NLRP3 sequence the showing conserved nature of putative palmitoylation sites across different species. **C)** Mouse NLRP3 AlphaFold protein structure depicting some of the putative Cys residues that can be palmitoylated in the LRR domain of the protein.

4.2.3 NLRP3 palmitoylation is conserved between mouse and humans.

As previously demonstrated, fatty acid synthesis, specifically the production of palmitate and subsequent palmitoylation, is essential for the activation of the NLRP3 inflammasome and downstream signalling. To elucidate the regulatory mechanisms, inflammasome reconstitution experiments were carried out in HEK293T cells. HEK293T cells were transiently transfected with plasmids expressing mouse or human NLRP3, ASC and caspase-1 (**Fig 4.3A-D**). HEK293T cells are unique as they lack endogenous expression of most immune receptors, including the NLRP3 inflammasome, thereby serving to address inflammasome assembly unaided by immune signalling. The cleavage of caspase-1 was observed in HEK293T cells reconstituted with inflammasome components; however, this cleavage was inhibited by the presence of cerulenin and 2BP (**Fig 4.3A and 4.3C**). Similar results were observed when HEK293T cells were instead reconstituted with plasmids expressing human inflammasome components. These findings suggest that the regulation of inflammasome by palmitoylation is conserved across species. Moreover, these data corroborate previous findings in this thesis, suggesting that the regulation of NLRP3 activation via FASN and palmitoylation occurs independently of any other immune signalling events.

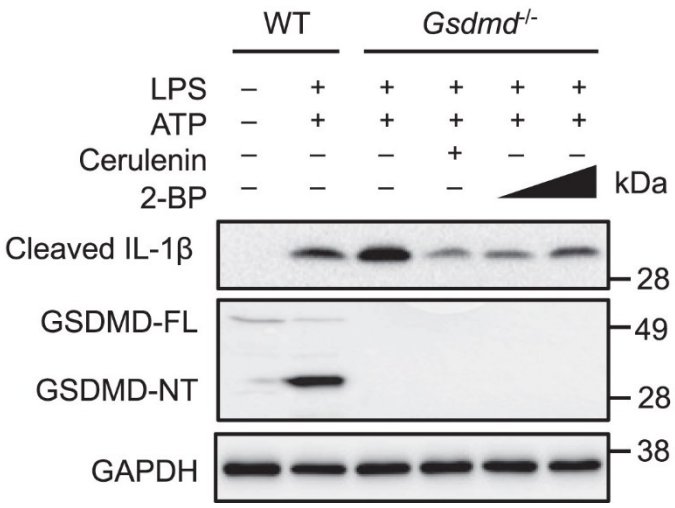


Figure 4.2: FASN production of palmitate has a distinct mechanism for regulation of the NLRP3 inflammasome. Immunoblots from WT and *Gsdmd*$^{-/-}$ iBMDMs exposed either to cerulenin (12.5 μM) or to palmitoylation inhibitor 2-BP (50 and 100 μM) and stimulated with the NLRP3 agonist ATP. Data shown are mean ± SD, and experiments shown are representative of at least three independent experiments.

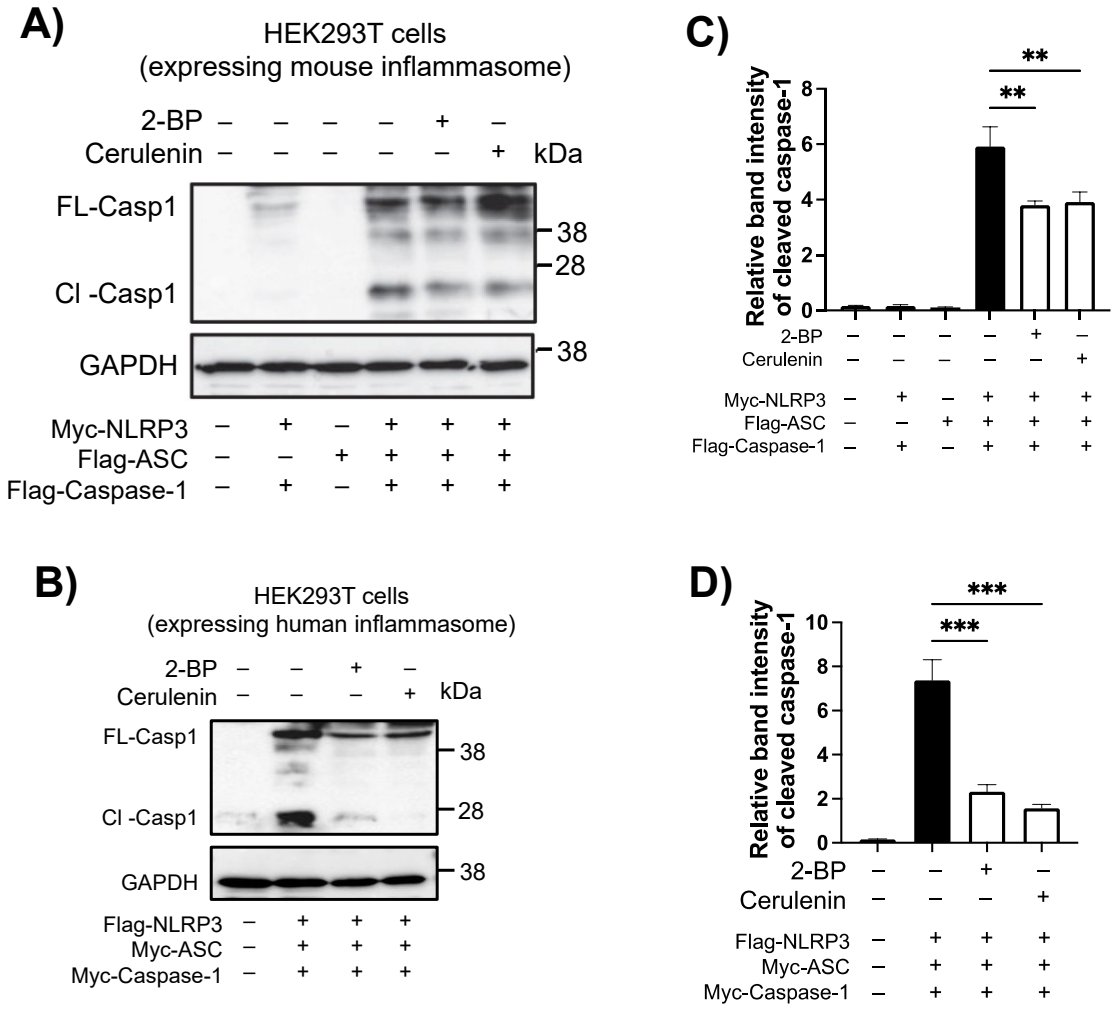


Figure 4.3: Fatty acid synthesis and palmitoylation are essential for oligomerisation of NLRP3. A) The NLRP3 inflammasome was reconstituted in HEK293T cells by transfecting with either the mouse protein-coding plasmids or **(B)** the human protein-coding plasmids. After 24h, cells were exposed to either cerulenin (12.5 μM) or 2-BP (100 μM) for 6h . Cell lysates were immunoblotted for caspase-1. (**C and D)** Relative band intensity of cleaved caspase-1 in A or B respectively**.** Experiments shown are representative of at least three independent experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$, by one-way ANOVA.

4.2.4 FASN and palmitoylation are essential for NLRP3 inflammasome assembly

To further elucidate the step at which NLRP3 inflammasome is regulated by the FASN pathway, NLRP3 ability for oligomerization was examined. To that end, the formation of ASC supramolecular complexes, termed ASC “specks,” was investigated. These complexes are detectable via microscopy and were observed in positive control cells exposed to NLRP3 activation signals (**Fig4.4A)**. Notably, treatment with palmitoylation inhibitors resulted in a significant reduction in ASC speck formation (**Fig 4.4A**). Quantification of these results was achieved by considering each cell as a data point examining several images and plotting the percentage of cells expressing a speck (**Fig 4.4B**). In this experiment, a sample size of 30 cells was selected as it was sufficient to capture representative biological variation while remaining feasible within the technical constraints. Our goal was to ensure that the data generated would be statistically meaningful, balancing both precision and practicality. Additionally, these experiments were conducted in triplicate to further support the observed findings and strengthen the validity of the results. Furthermore, the cells used are biologically homogeneous, allowing for a smaller population size to be effective for the observed effect to be noticed.

Previous studies have demonstrated that pyroptosomes in primed macrophages are predominantly associated with ASC dimers, which serve as the initial point for NLRP3 complex formation. In the current results, a complete loss of the ability to form dimers was observed when either the FASN or the downstream palmitoylation was inhibited (**Fig 4.3C**). Given that ASC is known to bind directly to pro-caspase-1, and that this interaction is essential for the cleavage of caspase-1 to its active form, the inability to form ASC specks is directly related to the reduction in caspase-1 cleavage previously observed.

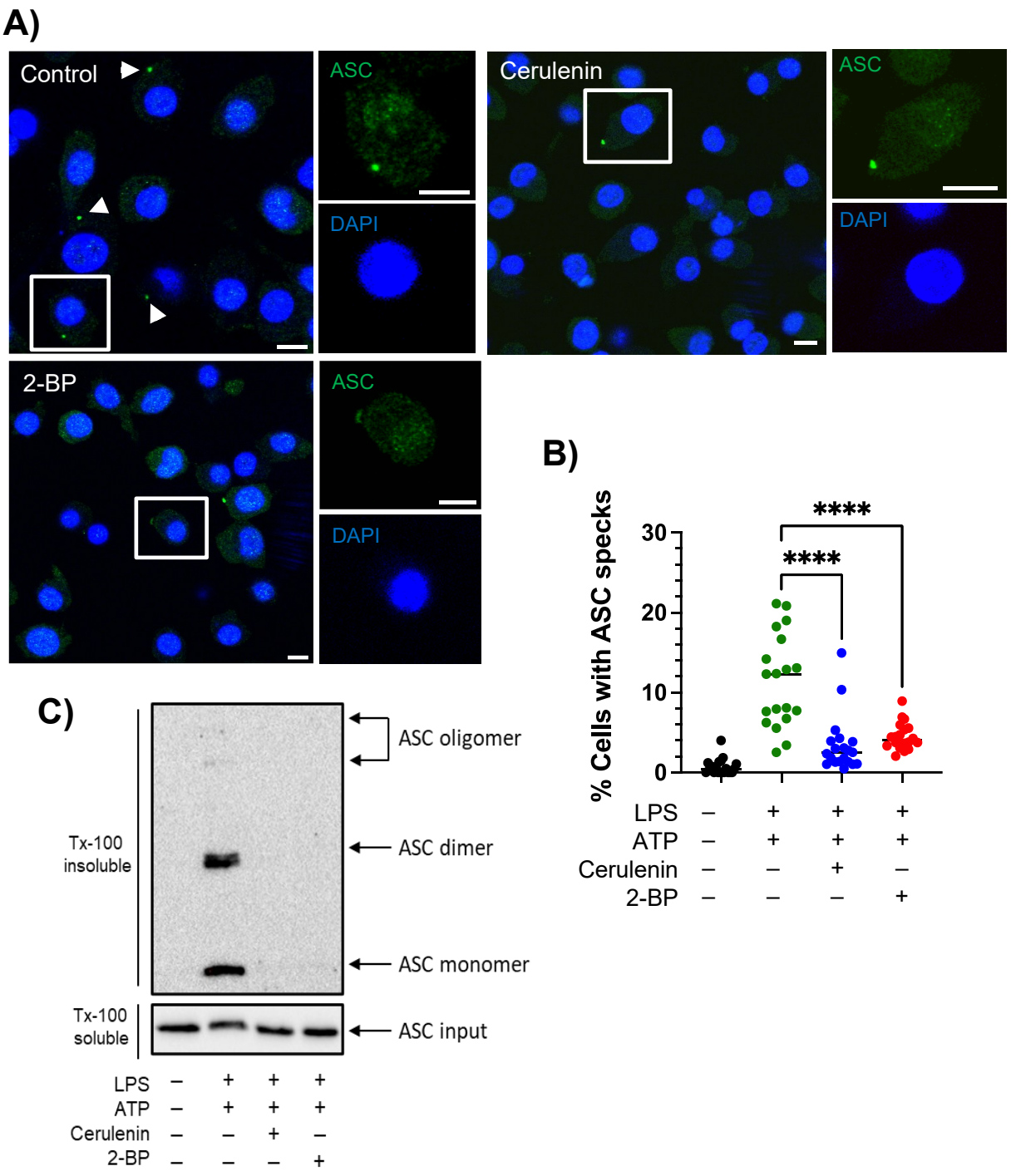


Figure 4.4: FASN is essential for assembly of the NLRP3 inflammasome complex *in vitro*
A) Immunofluorescent images from BMDMs exposed to LPS+ATP in the absence or presence of cerulenin (12.5 μM) or 2-BP (100 μM) to visualize ASC "specks." **B)** quantitative analysis of the percentage of cells with ASC specks. Each dot represents an individual field with at least $n = 30$ cells. **C)** ASC oligomerization in BMDMs exposed to LPS+ATP in the absence or presence of cerulenin (12.5 μM) or 2-BP (100 μM) with the initial lysate serving as an input control. Experiments shown are representative of at least three independent experiments. Scale bars, 5 μm; n.s., not significant. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$, by one-way ANOVA.

4.2.5 AIM2 inflammasome is not impacted by the inhibition of palmitoylation

Given the evidence that NLRP3 requires palmitoylation for ASC speck formation, the specificity of palmitoylation in regulating NLRP3 inflammasome assembly was undertaken. Previous findings indicated that while NLRP3 is dependent on palmitoylation for activation, AIM2 does not share this requirement. Microscopy was employed to observe ASC speck formation in AIM2-stimulated macrophages. Notably, upon AIM2 activation by dsDNA, a substantial percentage of cells depicted ASC specks formation. However, the formation of ASC specks remained unaltered when either FASN or palmitoylation was pharmacologically blocked (**Fig 4.5A**). Quantification of these results demonstrated no significant inhibition of ASC speck formation (**Fig 4.5B**). These findings support the hypothesis that FASN and palmitoylation uniquely regulate the NLRP3 inflammasome.

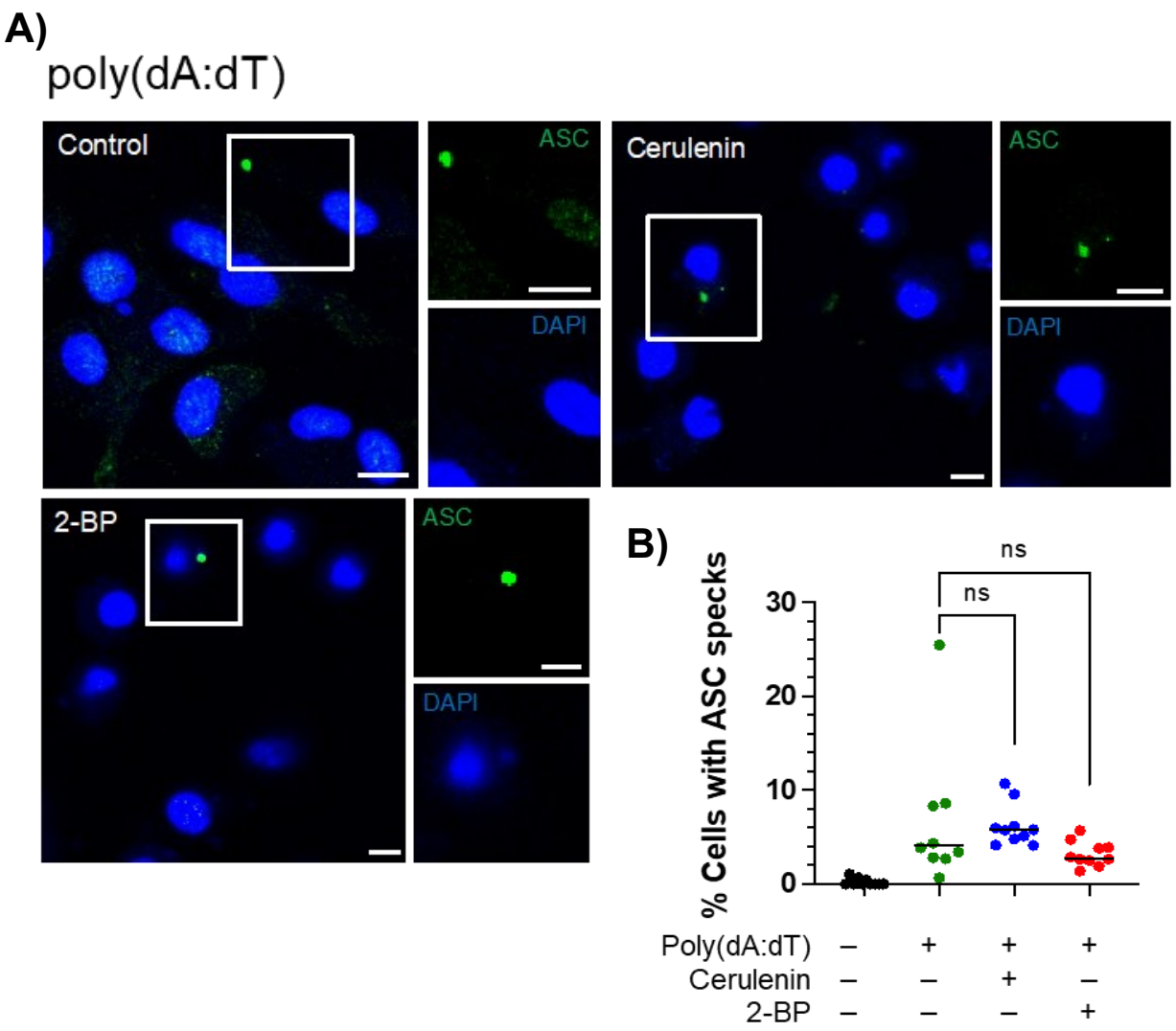


Figure 4.5: AIM2 inflammasome is not impacted by inhibition of palmitoylation. **A)** Immunofluorescent images of LPS-primed BMDMs transfected with poly(dA:dT) and treated with or without cerulenin (12.5 μM) or 2-BP (100 μM) to visualise ASC specks. **B)** Quantitative analysis of the percentage of cells with ASC specs. Each dot represents an individual field with at least *n* = 30 cells. n.s., not significant by one-way ANOVA. Scale bars, 5 μm.

4.2.6 Palmitoylation inhibits NLRP3 activation *in vivo*

With robust evidence supporting the role of palmitoylation as a regulator of NLRP3 inflammasome activation *in vitro*, confirmation of this process *in vivo* was sought. A murine peritonitis model was employed to investigate the physiological role of FASN and palmitoylation by employing a previously published protocol (Green *et al.*, 2020). C57BL6/J mice received intraperitoneal injections of the FASN inhibitor cerulenin, the palmitoylation inhibitor 2BP, or the NLRP3 inhibitor MCC950, alongside LPS. Following a 4-hour period, NLRP3 inflammasome activation was induced by the intraperitoneal injection of ATP. Animals were euthanized and peritoneal exudate cells and serum samples were collected 15 minutes post-ATP injection. ATP administration resulted in a significant increase in serum IL-1β levels in control animals, which was markedly reduced by treatment with the NLRP3 inhibitor MCC950 (**Fig 4.6A**). Inhibition of FASN activity or palmitoylation similarly resulted in reduced IL-1β levels, paralleling the effects observed with MCC950, indicating an NLRP3-dependent mechanism (**Fig 4.6A)**.

To assess corresponding caspase-1 activity *in vivo*, FAM-FLICA, a cell-permeable probe that covalently binds to active caspase-1, was utilised. NLRP3 inflammasome activation *in vivo* led to an increase in caspase-1 activity in $CD11b^{+}Gr1^{-}$ monocytes, as determined by flow cytometry of exudate cells. In contrast, caspase-1 activity was significantly diminished in mice treated with cerulenin or the palmitoylation inhibitor 2-BP (**Fig 4.6B**). These data unambiguously demonstrate that the FASN/palmitoylation axis is important for NLRP3 inflammasome activation both *in vitro* and *in vivo*.

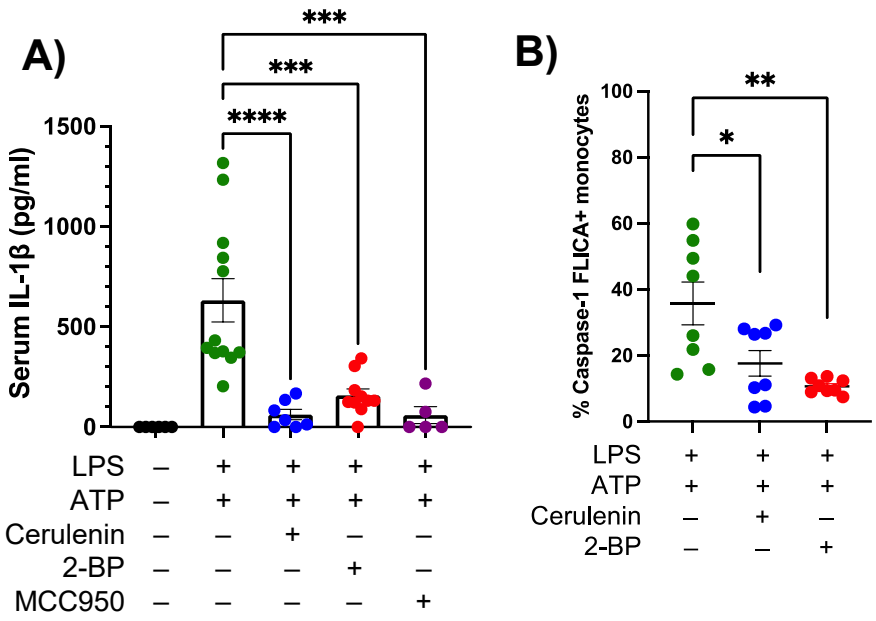


Figure 4.6: Palmitoylation inhibits NLRP3 activation *in vivo*. A) C57BL/6 mice were administered intraperitoneally (i.p.) with vehicle control (n = 12) or cerulenin (50 mg kg^{-1}; n = 7) or 2-BP (100 mg kg^{-1}; n = 10) or MCC950 (50 mg kg^{-1}; n = 5) and LPS (100 µg kg^{-1}). After 4h, mice were i.p. injected with ATP for 15 min. once collected L-1β was detected in the serum. **B)** Mice were stimulated as mentioned before with plot showing frequency of caspase-1 active CD11b+Gr1-monocytes in peritoneal exudate cells. Data shown are mean ± SD or mean ± SEM, Each dot represents a mouse. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$, by one-way ANOVA.

4.2.7 Validation of NLRP3 palmitoylation by acyl-biotin exchange assay

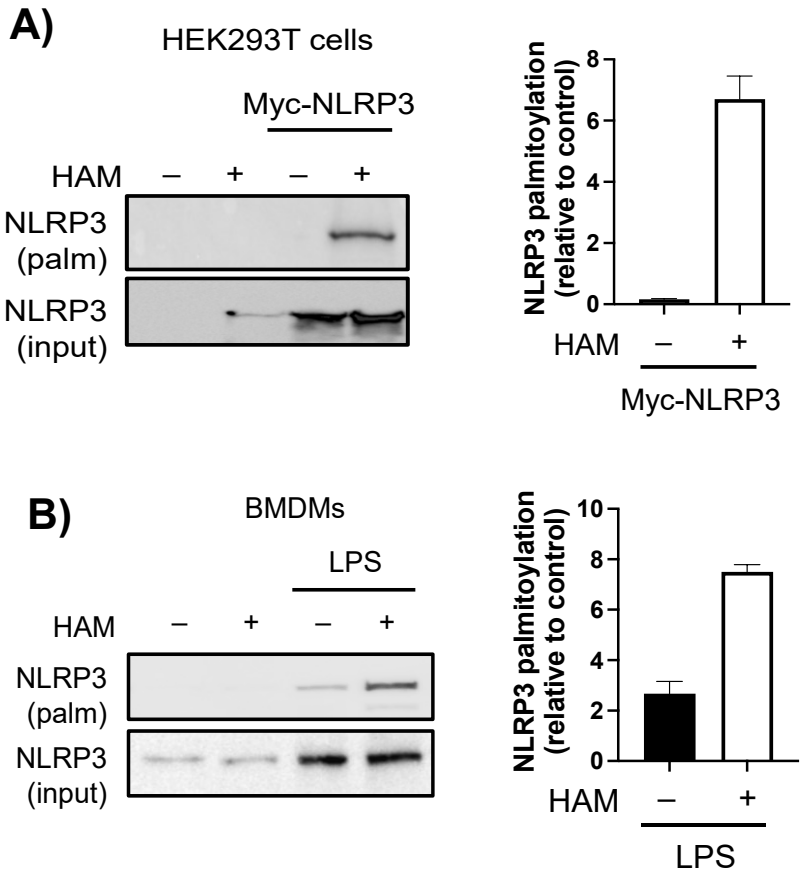
Having confirmed *in silico* that NLRP3 undergoes palmitoylation, a method for the detection of palmitoylated proteins was optimised using acyl-biotin exchange (ABE) assay (Leishman, Aljadeed and Anand, 2024). This semi-quantitative method allows for the identification of proteins that have been *S*-palmitoylated. Following treatment, cells were collected, and free cysteine sites were blocked with N-ethylmaleimide (NEM) to prevent false positives. Subsequently, palmitate-bound cysteine residues were cleaved with hydroxylamine (HAM), resulting in two sample fractions: hydroxylamine-treated (HAM)-positive and untreated, HAM-negative samples. In the HAM (+) samples, palmitate residues were cleaved and replaced with a thiol-reactive biotin group for affinity purification. The HAM (−) samples served as negative controls. Subsequently, affinity purification with biotin was performed, allowing selective pull-down of palmitoylated proteins using avidin-labelled agarose beads.

This enabled the identification of palmitoylated NLRP3. The protocol was validated using caveolin-1 (Cav-1) as a positive control (data shown in star protocol ref)(Leishman, Aljadeed and Anand, 2024). Immunoblot analysis confirmed the presence of *S*-acylated NLRP3 in the HAM (+) HEK293T samples expressing Myc-NLRP3, while no acylation was detected in the HAM (−) fraction or in cells lacking Myc-NLRP3 expression (**Fig 4.7A**). ABE assays were also performed in BMDMs to further validate endogenous NLRP3 palmitoylation. The assay confirmed that NLRP3 indeed undergoes palmitoylation in response to the priming signal LPS (**Fig 4.7B**).

To investigate whether priming or the activation signals promote NLRP3 palmitoylation, mouse macrophages were treated with LPS in the presence or absence of ATP, along with samples co-treated with either a FASN inhibitor or a palmitoylation inhibitor. Consistent with previous findings, LPS alone was found to induce significant NLRP3 palmitoylation, which was notably reduced in the presence of either cerulenin or 2-BP. In contrast, subsequent exposure to ATP did not enhance NLRP3 palmitoylation levels; rather, a slight reduction was observed, exhibiting minimal sensitivity to FASN and palmitoylation inhibitors (**Fig 4.7C**). Exposure to either pharmacological inhibitor in conjunction with the NLRP3 activation signal resulted in only a modest reduction in caspase-1 cleavage or IL-1β secretion (**Fig 4.7D, 4.7E**). These results suggest that palmitoylation predominantly occurs during the priming phase, while ATP has minimal impact on NLRP3 palmitoylation.

These results were further confirmed *in vivo* using C57BL6/J mice, which were stimulated as previously described. An ABE assay conducted on thioglycollate-elicited peritoneal cells, a common source of peritoneal macrophages, obtained from mice injected with LPS and ATP revealed the presence of palmitoylated NLRP3. This palmitoylation was diminished in cells

from animals additionally treated with either the FASN or the palmitoylation inhibitor (**Fig 4.7F**). By demonstrating that NLRP3 palmitoylation occurs *in vivo*, these results demonstrate that NLRP3 palmitoylation is an essential requirement for NLRP3-mediated inflammatory response.


A)
HEK293T cells
Myc-NLRP3
HAM – + – +
NLRP3 (palm)
NLRP3 (input)
NLRP3 palmitoylation (relative to control)
0
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4
6
8
HAM – +
Myc-NLRP3
B)
BMDMs
LPS
HAM – + – +
NLRP3 (palm)
NLRP3 (input)
NLRP3 palmitoylation (relative to control)
0
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HAM – +
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C)
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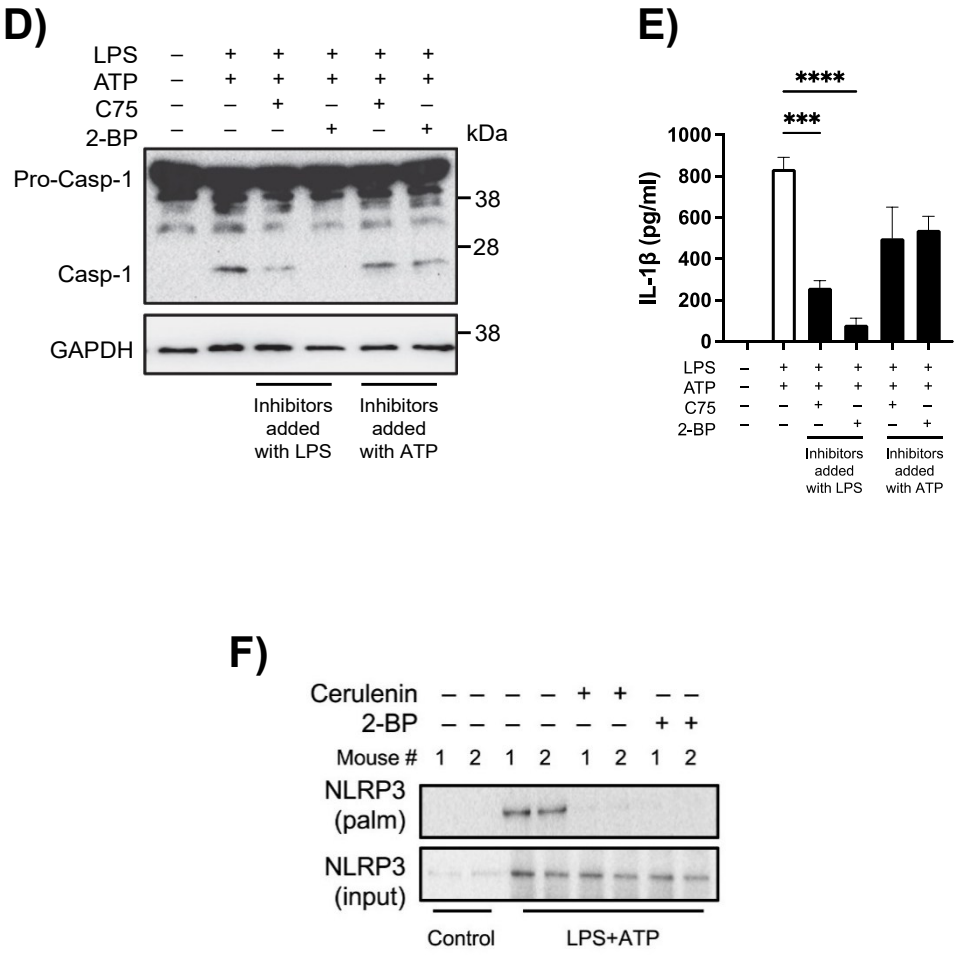


Figure 4.7: ABE assay elucidates more about NLRP3 reliance on palmitoylation. A) HEK293T cells were either transfected with wild-type Myc-NLRP3 or left untransfected. After 24 hours, cell lysates were divided into HAM (+) and HAM (−) groups. Palmitoylation levels of NLRP3 were assessed using the ABE assay, followed by immunoblotting with an anti-NLRP3 antibody. Total NLRP3 levels were shown as input controls. Right: quantification of the relative intensity of palmitoylated NLRP3 bands**. B)** Immunoblot analysis of palmitoylated NLRP3 and total NLRP3 (input control) in BMDMs treated or untreated with LPS (500 ng/mL, 3.5 h), using the ABE assay. Right: relative band intensity of palmitoylated NLRP3. **C)** Immunoblots of palmitoylated and total NLRP3 (input control) in iBMDMs treated with LPS alone or LPS + ATP, with concurrent treatment using cerulenin or 2-BP. Right: relative band intensity of palmitoylated NLRP3. **(D, E)** Cells treated with the same inhibitors as in **(C)** were probed for caspase-1 and GAPDH by immunoblotting. **(E)** Levels of secreted IL-1β in the supernatant were measured. **F)** Thioglycolate-elicited peritoneal cells from treated animals were subjected to the ABE assay to determine palmitoylated NLRP3 levels. Total NLRP3 in the initial lysates served as input controls. Right: relative band intensity of palmitoylated NLRP3. All experiments were repeated at least three times independently. Statistical significance: n.s. (not significant), $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$, by one-way ANOVA.

4.2.8 NLRP3 Cys898 palmitoylation is critical for inflammasome activation

To pinpoint the specific cysteine residue in NLRP3 that undergoes palmitoylation, three putative sites—Cys671, Cys835, and Cys898—were selected for investigation. These sites are located in the LRR region and are conserved across multiple species. In the in silico analysis, these sites showed a high predicted score for palmitoylation. To keep experimental constraints manageable, these sites were chosen for simplicity; however, future work should consider investigating other LRR-based palmitoylation sites. Each residue was individually mutated to alanine in Myc-tagged NLRP3 expression constructs, which were then analysed using an ABE assay alongside Myc-NLRP3 WT in HEK293T cells. As expected, the WT NLRP3 exhibited robust palmitoylation. Notably, mutating Cys898 to alanine (C898A) caused a significant reduction in NLRP3 palmitoylation (**Fig. 4.8A**). In contrast, the C671A mutation led to a slight but non-significant increase in palmitoylation, while the C835A mutation resulted in a modest decrease in palmitoylation levels. Additionally, in an inflammasome reconstitution assay, the NLRP3 C898A mutant showed a marked reduction in caspase-1 activation compared to NLRP3 WT (**Fig. 4.8B**). In contrast, the NLRP3 C671A mutation exhibited caspase-1 activation comparable to NLRP3 WT in HEK293T cells. Together, these findings highlight Cys898 as a critical site for NLRP3 palmitoylation and inflammasome activation.

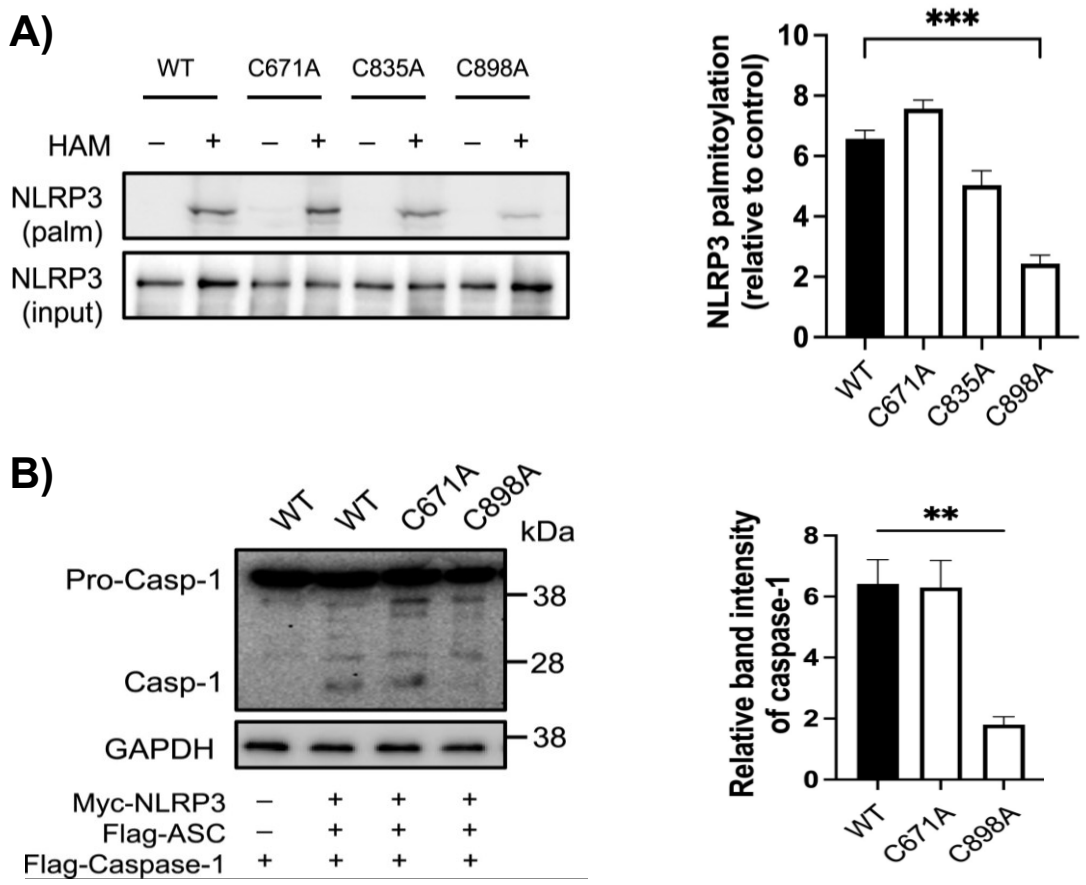
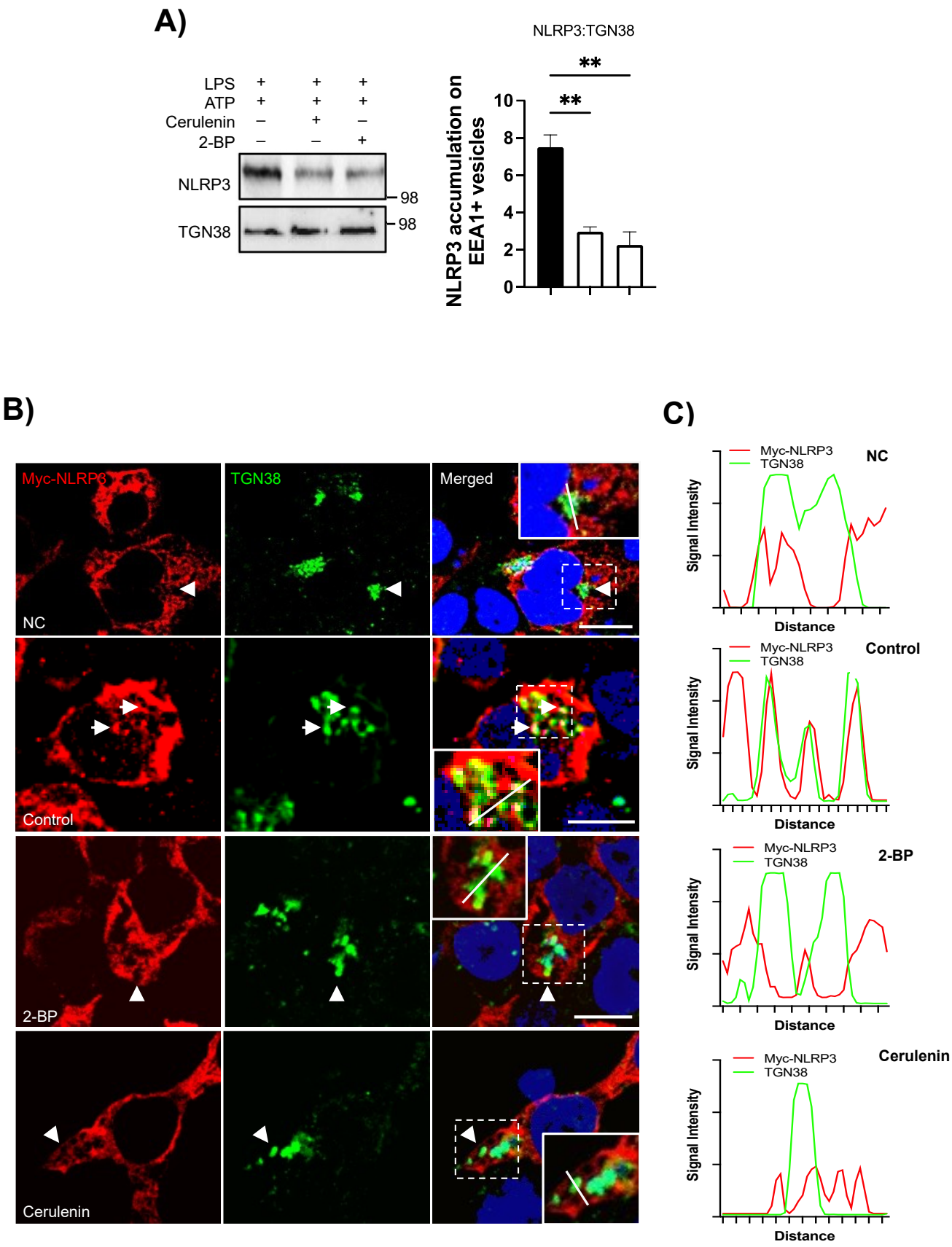


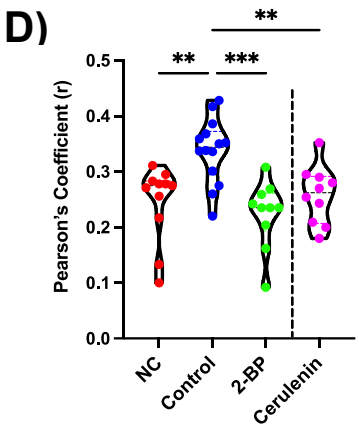
Figure 4.8: NLRP3 Cys898 palmitoylation is critical for inflammasome activation. **A)** Immunoblots showing palmitoylated and total NLRP3 (input control) in HEK293T cells transfected for 24 hours with either wild-type Myc-NLRP3 or the C671A, C835A, or C898A mutants, analysed using the ABE assay. Relative band intensity of palmitoylated NLRP3 for each construct is quantified. **B)** The NLRP3 inflammasome was reconstituted in HEK293T cells by transfection with the indicated Myc-NLRP3 constructs. Cell lysates were analysed for caspase-1 activation via immunoblotting, with quantification of cleaved caspase-1 intensity. Data are presented as mean ± SD. **p < 0.01; ***p < 0.001, using one-way ANOVA for statistical analysis.

4.2.9 Palmitoylation licenses NLRP3 translocation to the TGN38 positive vesicles

As previously noted, recent studies have demonstrated that NLRP3 associates with dTGN via its polybasic sequence, with membrane association being essential for the formation of the NLRP3 double-ring cage structure (Chen and Chen, 2018b; Andreeva *et al.*, 2021). More recent research has shown that the vesicles to which NLRP3 localises are actually of endosomal origin. These vesicles are positive for both the early endosomal marker EEA1 and the Golgi transport protein TGN38 (known as TGN46 in humans) (Lee *et al.*, 2023; Zhang. *et al.*, 2023). However, the polybasic region alone is typically insufficient for strong membrane association due to weaker ionic binding; an additional lipid anchor is required. Therefore, it was hypothesised that palmitoylation is necessary for the endosomal accumulation of NLRP3 upon inflammasome activation.

To test this hypothesis, endosomes were purified from mouse macrophages using a polyclonal antibody against the endosomal marker early endosome antigen 1 (EEA1). As anticipated, immunoblotting confirmed substantial TGN38 expression in the purified endosomes (**Fig 4.9A**). Consistent with previous reports, endosomes from cells treated with LPS and ATP exhibited significant NLRP3 accumulation. In contrast, endosomes from cells treated with either FASN or palmitoylation inhibitors displayed reduced NLRP3 recruitment. These findings solidify the conclusion that NLRP3 localisation to endosomal sites is mediated by palmitoylation.


A)
LPS + + +
ATP + + +
Cerulenin – + –
2-BP – – +
NLRP3
TGN38
98
98
NLRP3:TGN38
NLRP3 accumulation on EEA1+ vesicles
**
**
10
8
6
4
2
0
B)
Myc-NLRP3
TGN38
Merged
NC
Control
2-BP
Cerulenin
C)
Myc-NLRP3
TGN38
NC
Signal Intensity
Distance
Control
2-BP
Cerulenin


D)
Pearson's Coefficient (r)
0.5
0.4
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0.2
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0.0
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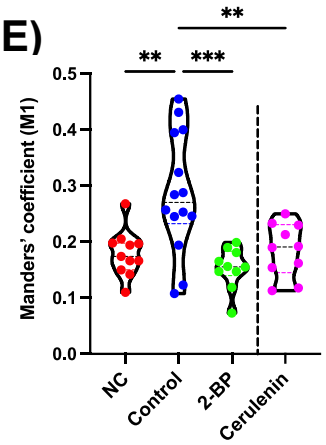
NC
Control
2-BP
Cerulenin


E)
Manders' coefficient (M1)
0.5
0.4
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0.2
0.1
0.0
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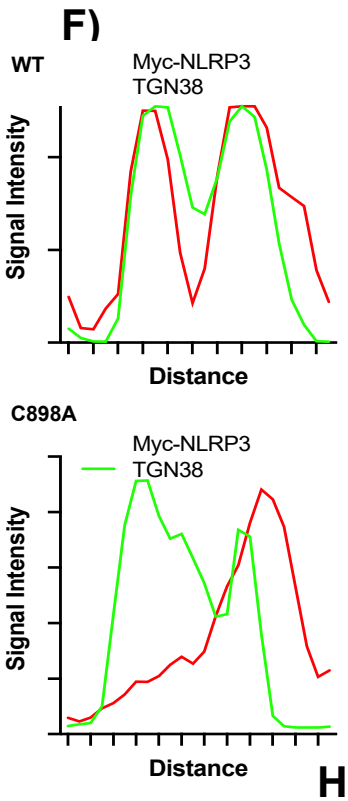
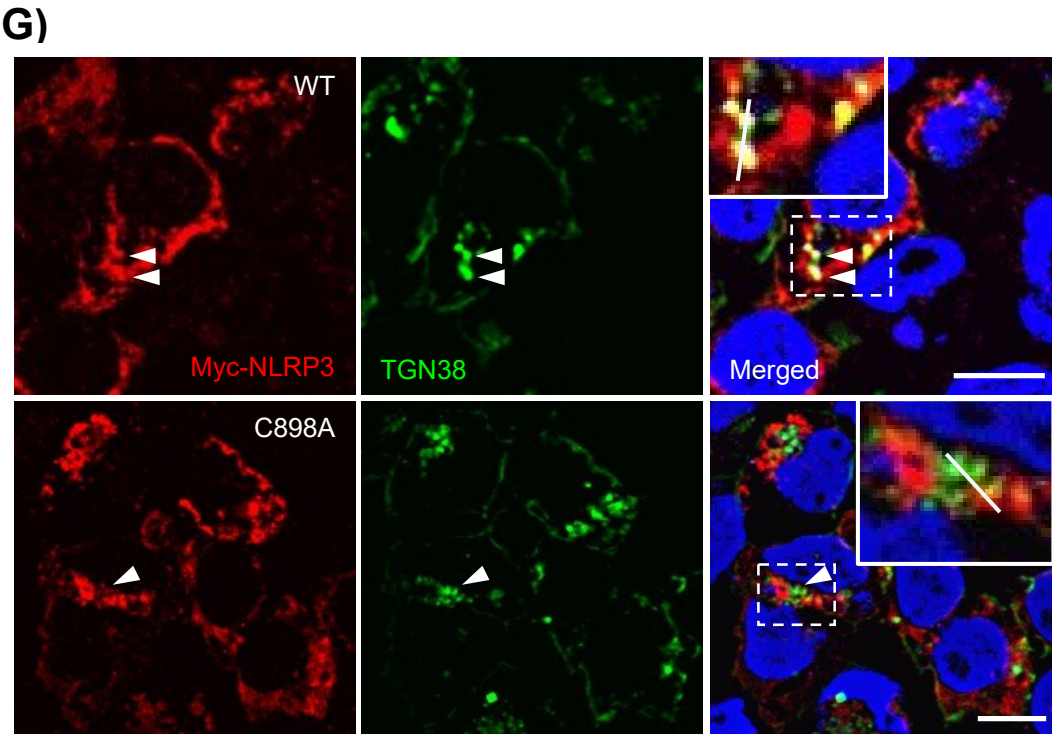
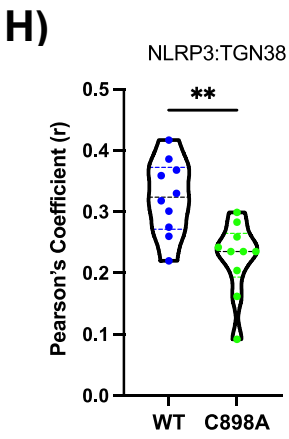
***
NC
Control
2-BP
Cerulenin


F)
WT
Myc-NLRP3
TGN38
Signal Intensity
Distance
C898A
Myc-NLRP3
TGN38
Signal Intensity
Distance


G)
WT
Myc-NLRP3
TGN38
Merged
C898A


H)
NLRP3:TGN38
Pearson's Coefficient (r)
0.5
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C898A

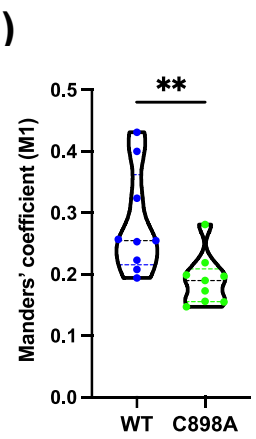

I)
Manders' coefficient (M1)
0.5
0.4
0.3
0.2
0.1
0.0
**
WT
C898A

Figure 4.9: Palmitoylation licenses NLRP3 translocation to the TGN vesicles. A) Immunoblot analysis of purified EEA1-positive endosomes from iBMDMs treated with LPS and ATP, with or without the presence of cerulenin or 2-BP. The relative band intensity of NLRP3 was normalised to TGN38 (right). **B)** HEK293T cells were transfected with Myc-NLRP3 and incubated for 24 hours, and either unstimulated (NC) or stimulated with ATP (control) added for the final hour following a 6-hour treatment with the palmitoylation inhibitor 2-BP or the FASN inhibitor cerulenin. Cells were fixed and stained with secondary antibodies. Key features are indicated by white arrows. **C)** Fluorescence intensity profiles were measured along the white bars shown in the insets in B, at a distance to ensure adequate comparison. **D)** Quantitative analysis of colocalization between Myc-NLRP3 and TGN38 using Pearson's correlation coefficient analysed using ImageJ. **E)** Quantitative measurement of the fraction of Myc-NLRP3 colocalising with TGN38, calculated using Manders' coefficient (M1) analysed using ImageJ. **(F ,G)** HEK293T cells were transfected with either wild-type Myc-NLRP3 (WT) or the C898A mutant and incubated for 24 hours, with ATP added for the last hour. **F)** Fluorescence intensity profiles were measured along the white bars in the insets shown in G. **H)** Quantitative representation of colocalization between Myc-NLRP3 and TGN38 using Pearson's correlation coefficient. **I)** Quantitative measurement of the fraction of Myc-NLRP3 overlapping with TGN38 using Manders' coefficient (M1). For all analyses, approximately 10-20 random fields were examined, with at least n = 20 cells per field. For co-localisation n=20. All experiments were repeated at least three times independently. Scale bars: 5 μm. Statistical significance: n.s. (not significant), $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, by one-way ANOVA.

To further investigate, HEK293T cells were transfected with Myc-tagged NLRP3, and its colocalization with the TGN38 positive endosomes was analysed. In control cells, Myc-NLRP3 was diffusely distributed throughout the cytosol and exhibited minimal overlap with the TGN38 marker (**Fig 4.9B-9E**). However, upon ATP stimulation, the TGN appeared to fragmented into multiple vesicles, this is expected of TGN38 cycle arrest. This demonstrated strong colocalization between Myc-NLRP3 and TGN38 positive endosomes. Treatment with either 2BP or cerulenin significantly reduced Myc-NLRP3 colocalization with TGN38 positive endosomes. To quantify these results, both Pearson's and Manders' correlation coefficients were applied. Calculations were performed using ImageJ, where regions of interest (ROIs) were selected based on signal presence. Background subtraction and thresholding were applied prior to colocalization analysis. Pearson's coefficient was used to assess the linear correlation between the two signals, while Manders' coefficient quantified the fraction of NLRP3 colocalising with TGN38-positive endosomes. Analyses were repeated multiple times; however, the results shown in the figure correspond to the region marked by the white line across the inset. The use of both methods enabled an evaluation of both the spatial overlap and the intensity relationship between the two signals.

Subsequently, the role of the Cys898 site in NLRP3 palmitoylation was examined by overexpressing Myc-tagged NLRP3 WT and mutant constructs in HEK293T cells. Cells expressing NLRP3 WT displayed strong colocalization with TGN38 positive endosomes following ATP stimulation, while the NLRP3 C898A mutant (**Fig 4.9F-I)** exhibited markedly reduced colocalization with TGN38 positive endosomes. Correspondingly, the fraction of NLRP3 C898A co-localising with TGN38 positive endosomal vesicles was significantly lower than that observed in cells expressing NLRP3 WT. These findings identify Cys898 as a critical site for NLRP3 palmitoylation and establish a mechanistic link between NLRP3 palmitoylation and its association with TGN38 positive endosomes.

4.3 Discussion

This chapter builds upon the previous findings in this thesis, establishing a relationship between lipid biosynthesis, palmitoylation, and NLRP3 activation. *In silico* methods were used to identify putative palmitoylation sites in NLRP3 inflammasome components. These approaches led to the conclusion that only the NLRP3 protein, but not ASC and caspase-1, carried palmitoylation sites. Further experiments validated that NLRP3 is indeed palmitoylated and that palmitoylation is critical for inflammasome assembly. Inhibition of palmitoylation not only reduced ASC speck formation but also the translocation of NLRP3 to TGN38-positive vesicles. Notably, the requirement for palmitoylation was limited to activation of the NLRP3 inflammasome. Consequently, inhibition of FASN or the process of palmitoylation failed to restrain AIM2 inflammasome activation. Moreover, palmitoylation was found critical for inflammasome assembly both *in vitro* and *in vivo*. In a mouse model of peritonitis, the cells recruited to the peritoneal cavity were sensitive to cerulenin and 2-BP when assayed for palmitoylation. Furthermore, there were fewer caspase-1-positive monocytes, and this was associated to overall reduced serum IL-1β levels in animals exposed to cerulenin and 2-BP compared to animals exposed only to LPS and ATP.

It should be noted that there are distinct differences between human and mouse inflammasome components, particularly in their response to FASN inhibitors like cerulenin and 2-BP. These inhibitors strongly inhibit caspase-1 cleavage in HEK293T cells expressing the human inflammasome but have a lesser effect in mouse cells. This discrepancy could be due to species-specific differences in inflammasome components, such as NLRP3, ASC, and caspase-1, as well as differences in how metabolic pathways, including fatty acid synthesis, are regulated (Heil *et al.*, 2004). These differences highlight the importance of considering species-specific variations when interpreting the effects of pharmacological inhibitors in inflammasome research. Future work would be benefit from using both human and mouse knockouts in conjunction to account for any variation that might be occurring between human and mouse NLRP3 regulation by FASN.

Recent findings demonstrated that NLRP3 assembles in TGN38-positive vesicles associated with the dTGN (Chen and Chen, 2018). However, this view has been contested. It was instead observed that the TGN38-positive vesicles are endosomal in nature (Lee *et al.*, 2023; Zhang. *et al.*, 2023). Using purified endosomes, we observed reduced NLRP3 accumulation when cells were treated with FASN or palmitoylation inhibitors, highlighting the role of palmitoylation in NLRP3 recruitment for inflammasome assembly. Further analysis in HEK293T cells revealed strong colocalization of NLRP3 with TGN38 after ATP stimulation, which was diminished with 2-BP or cerulenin treatment. Similarly, mutating the NLRP3 Cys898

palmitoylation site also reduced NLRP3 colocalization with TGN38, identifying Cys898 residue as critical for NLRP3 palmitoylation and dTGN vesicle association.

Palmitoylation is the covalent attachment of palmitic acid to proteins, typically at cysteine residues via the formation of thioester bonds, and is catalysed by enzymes called palmitoyl acyltransferases (PATs) (Mitchell *et al.*, 2006). There are 23 known PAT enzymes in mammals characterized by the presence of a conserved cysteine-rich domain (CRD) containing a DHHC (Asp-His-His-Cys) motif (Putilina, Wong and Gentleman, 1999). Palmitoylation plays a crucial role in intracellular signalling, serving as a regulatory mechanism that controls protein trafficking and localisation (Ernst *et al.*, 2018). A recent study demonstrated that the loss of acyltransferase zDHHC12 leads to an increase in the severity of responses to endotoxin shock models, as this acyltransferase targets Cys844 on the NLRP3 protein (Wang *et al.*, 2023). From this study, it was concluded that palmitoylation dampens NLRP3 inflammasome activation and diminishes the secretion of IL-1β. Here, palmitoylation acted to promote NLRP3 degradation through chaperon-mediated autophagy. This is in direct contradiction to a number of studies demonstrating heightened inflammasome activity in relation to palmitate exposure, without affect NLRP3 protein levels (Wen *et al.*, 2011b; Shirasuna *et al.*, 2016; Korbecki and Bajdak-Rusinek, 2019; Sano *et al.*, 2020). In the study revealing Cys844 palmitoylation, the inflammasome experiments were limited to *zDHHC12*$^{-/-}$ cells, where acyltransferase loss may broadly affect protein stability, localisation, and interactions beyond NLRP3. Although their model conflicts with the data reported in this thesis (and other recently published studies), palmitoylation may regulate NLRP3 at different stages or in specific contexts, explaining their distinct observations.

Another recent study published by Zheng *et al* showed that zDHHC5 was able to palmitoylate the Cys838 residue on NLRP3, leading to enhanced NLRP3-NEK7 interaction and inflammasome activation (Zheng *et al.*, 2023). NEK7 is an essential protein that interacts with NLRP3 and facilitates the inflammasome platform assembly (He *et al.*, 2016). The study by Zheng *et al.* displayed a positive relationship between palmitoylation and NLRP3 activation, though the distinction in the site of palmitoylation lends itself to the idea that palmitoylation is a complex regulatory mechanism. It is plausible that NLRP3 palmitoylation mirrors other PTMs such as phosphorylation and ubiquitination, where the upstream enzymes involved and the palmitoylation site are stimuli-dependent.

In the current thesis, palmitoylation was observed to influence inflammasome assembly independently of any effect on GSDMD that has also been recently shown to be palmitoylated (Du *et al.*, 2024; Zhang *et al.*, 2024). In the study by Balasubramanian et al., it was shown that palmitoylation of GSDMD at the Cys191/192 residues (human/mouse) influences the

membrane translocation of the N-terminal fragment of GSDMD and subsequent pore formation. The authors identified that palmitoylation of GSDMD is dependent on zDHHC5 and zDHHC9, and that this modification is induced by LPS-induced reactive oxygen species (Balasubramanian *et al.*, 2024). Interestingly, these findings were corroborated and expanded upon by Zhang *et al* that not only demonstrated that GSDMD was palmitoylated during pyroptosis but that upon translocation to the plasma membrane the N-terminal GSDMD was then de-palmitoylated by the acyl protein thioesterase enzyme, which allowed for pore formation (Zhang *et al.*, 2024). These findings demonstrated the variety of proteins in the inflammasome pathway that are regulated by palmitoylation. Additionally, they highlight the significance of palmitoylation and depalmitoylation cycles as has been previously reported for other cellular processes (Fukata *et al.*, 2013).

Palmitoylation is not the only lipid-dependent PTM that has been implicated in metabolic conditions**.** Malonylation, a process whereby malonyl is attached to lysine residues on proteins, is closely tied to the metabolic state of cells, with increased levels of malonyl-CoA enhancing protein malonylation (Zou *et al.*, 2023). Several substrates for malonylation are known, including GAPDH and histone proteins, and these may regulate inflammatory activity (Galván-Peña *et al.*, 2019; R. Zhang *et al.*, 2023). Given recent reports that lipid metabolism tightly regulates the inflammasome, it is tempting to speculate the role of other such lipid-dependent modifications including prenylation and farnesylation in NLRP3 inflammasome activation. However, a quick *in silico* approach failed to reveal any such sites in NLRP3 inflammasome components.

Palmitoylation has also been implicated in disease models. Palmitoylation of CD36, a widely expressed membrane glycoprotein that modulates fatty acid uptake and β-oxidation, has been shown to influence fatty acid homeostasis (Samovski *et al.*, 2015). This work identified CD36 palmitoylation to facilitate fatty acid uptake and lipid accumulation, through reduced fatty acid oxidation. This led to increased susceptibility to non-alcoholic steatohepatitis (NASH) in a mouse model of the disease. Intriguingly, *Cd36*-deficient animals, expressing CD36 lentiviral constructs with mutated palmitoylation site, demonstrated reduced NASH compared to control animals expressing WT CD36 constructs. In the current thesis, pharmacological inhibition of FASN resulted in reduced serum IL-1β levels in a mouse model of peritonitis. It would be interesting to test above approaches in *Fasn*-deficient animals to gain valuable insights. However, FASN is pivotal during development and *Fasn*$^{-/-}$ animals are nonviable. To overcome this limitation, animal models with FASN deficiency in the myeloid compartment could be utilised to examine the physiological significance of FASN-mediated palmitoylation in NLRP3-dependent diseases.

Building upon the findings presented in Chapter 3 that established a clear link between FASN regulation of NLRP3, this chapter identified a novel PTM by which FASN calibrates NLRP3 inflammasome activation. These results provide further insight into the intricate relationship between the lipid metabolism and NLRP3, contributing to a deeper understanding of how palmitoylation influences inflammasome activity. This highlights the complexity of lipid-mediated regulation in immune responses.

Results

Chapter 5

Untargeted lipidomics of the canonical and non-canonical NLRP3 inflammasome

Chapter 5. Untargeted lipidomics of the canonical and non-canonical NLRP3 inflammasome

5.1 Introduction

Lipids, once considered merely as structural components of cell membranes and energy storage molecules, are now recognised as critical mediators of various biological processes, including signal transduction, membrane dynamics, and cellular homeostasis (Chamberlain and Shipston, 2015; Sunshine and Iruela-Arispe, 2017; Cockcroft, 2021). Analysis of the lipidome has revolutionised our understanding of the complexity and diversity of lipids within biological systems, offering insights into their multifaceted roles in cellular function and disease (Géhin, Fowler and Trivedi, 2023). The variety of roles is underscored by the sheer vastness of lipids within the lipidome (Munro, 2003; Shimizu, 2009; Anand, 2020). The lipidome, encompassing thousands of lipid species with distinct structural variations, presents both an analytical challenge and a vast opportunity to gain new biological insights (Anand, 2020). The primary issues faced in studying the lipidome are the ephemeral nature of lipids, their tendency to be easily influenced by a wide number of stimuli, and their unstable nature during sample storage and preparation (Murphy, 2018). Whilst studying the lipidome at homeostasis is difficult, attempting to understand the lipidome during the dynamic activation of NLPR3 is even more complex, as there is only a short period of time in which degradation of lipids and pyroptosis of the cell occurs (Andersen, 2022). Rising to this challenge, liquid chromatography coupled with mass spectrometry (LC-MS) is a highly advanced tool which can address several of these issues while offering unparallel insights into metabolic rewiring during NLRP3 activation.

LC-MS offers a precise approach for detecting both predefined and unknown targets (Macklin, Khan and Kislinger, 2020; Dueñas *et al.*, 2023; Salihovic *et al.*, 2023). It combines powerful analyte identification capabilities with stringent requirements for accuracy (Olshina and Sharon, 2016). In lipid analysis, preparation begins with liquid-liquid or supported liquid extraction to remove contaminants, followed by chromatography to separate analytes by properties like polarity, aiding in distinguishing similar compounds (Li and Bartlett, 2014; Coskun, 2016). Electrospray ionisation (ESI) adds charges to analytes for mass-to-charge ratio analysis, while tandem MS (MS-MS) provides further structural insights (Glish and Vachet, 2003).

Mass spectrometry has quantitative and qualitative applications, with the latter increasingly used to explore unknown analyte landscapes (Lietz, Gemperline and Li, 2013). Although most research focuses on predefined targets, untargeted approaches, such as desorption

electrospray ionisation mass spectrometry (DESI-MS), are uncovering novel biomarkers. DESI-MS has been used in oncology to map lipid distributions in tumours, tumour margins, and healthy tissues, aiding cancer diagnosis (Takáts *et al.*, 2004; Calligaris *et al.*, 2014; Holzlechner, Eugenin and Prideaux, 2019; Souza and Patti, 2021; Kumar, 2023). Initially developed for toxicological studies, mass spectrometry is now integral to diagnostics, including endocrine disorders like polycystic ovary syndrome, where steroid hormone quantification is critical (Ketha, Singh and Ketha, 2017; Allen and McWhinney, 2019; Macklin, Khan and Kislinger, 2020). These advances have improved testing sensitivity and population identification. However, the potential for mass spectrometry to decipher upstream lipidome shifts in disease remains largely untapped.

With the rising prevalence of metabolic disorders linked to NLRP3 inflammasome activation, high-throughput and highly specific detection techniques, such as LC-MS, show great promise as diagnostic tools (Duran-Salgado, 2014; Krishnan *et al.*, 2016; Mortimer *et al.*, 2016). It can be applied to a range of tasks, such as characterising the sequence of NLRP3 and identifying PTMs via analysing the peptides derived from NLRP3. For instance, studies like Stutz et al. (2017) used peptide digestion of NLRP3 to identify novel phosphorylation sites in the pyrin domain, showing that introducing phosphomimetic residues at these novel sites abrogated NLRP3 activation (Stutz *et al.*, 2017). LC-MS has also been employed to investigate the role of lipid changes during NLRP3 activation. Rampanelli et al. (2018) used electrospray ionisation tandem mass spectrometry (ESI-MS/MS) to analyse lipid profiles in a model of metabolic injury induced by LDL-mediated lipotoxicity in renal tubular cells. Their findings showed that inflammasome activation led to notable lipid changes while NLRP3 knockout mice exhibited minimal lipid accumulation, underscoring the role of NLRP3 in lipid metabolism during inflammation (Rampanelli *et al.*, 2018). These studies highlight the versatility of mass spectrometry in uncovering new pathways that regulate inflammation. NLRP3 inflammasome activation is a dynamic process, with multiple triggers and regulatory mechanisms that can be explored further through this advanced analytical technique (Chu *et al.*, 2018; Swanson, Deng and Ting, 2019; Y. Z. Zhang *et al.*, 2019; Olona, Leishman and Anand, 2022).

The NLRP3 inflammasome can also be induced by a non-canonical pathway, which is mediated by caspase-11 in mice and pro-caspase-4/5 in humans (Ghait *et al.*, 2023). Whereas in the canonical inflammasome there is a priming and activation signal, the non-canonical inflammasome has low basal expression of pro-caspase-11/4/5, which is upregulated in the response to IFN-y binding (Viganò *et al.*, 2015). Once expressed, these caspases can directly bind to cytosolic LPS and undergo autoproteolytic cleavage to their active form, at which point they are able to act in a similar manner to the canonical inflammasome (Broz and Dixit, 2016). More than just static PRRs, the non-canonical inflammasomes has been suggested to directly

target pathogen-containing phagosomes through actin-interacting proteins and RhoA, promoting bacterial clearance (Caution *et al.*, 2015). Overall, these studies demonstrate that non-canonical NLRP3 plays a crucial role in host defence.

Whilst not as versatile as the canonical NLRP3 pathway, the non-canonical inflammasome presents a potential therapeutic target, including for highly variable, naturally occurring prostaglandin E2 (PGE2), which is hypothesised to exhibit both pro- and anti-inflammatory properties in human asthma (Zasłona *et al.*, 2020; Cahoon, Yang and Wang, 2022). Recent research has shown that PGE2 can inhibit the NLRP3 inflammasome through both extracellular and intracellular mechanisms, suggesting a connection to both canonical and non-canonical pathways (Sokolowska *et al.*, 2015). For instance, extracellular PGE2 affects upstream priming signals typically associated with the canonical inflammasome, while intracellularly it may modulate caspase-11 activity crucial to the non-canonical pathway. LC-MS has been instrumental in these discoveries, enabling precise quantification and profiling of PGE2 in biological samples (Sokolowska *et al.*, 2015). This dual regulation makes PGE2 an appealing target for pharmacological intervention to mitigate the severity of asthma attacks (Downs *et al.*, 2020).

In this chapter, the lipidome changes that occur during the canonical and the non-canonical NLRP3 inflammasome have been investigated. This analysis was conducted through untargeted lipidomics providing a comprehensive view of the lipidome and identifying lipid classes involved in NLRP3 inflammasome activation.

5.2 Results

5.2.1 Overview of mass spectrometry workflow and confirmation of inflammasome activation

Leukocyte fractions were isolated from human whole blood and stimulated with M-CSF to induce differentiation into human monocyte-derived macrophages (hMDMs). Samples from six healthy donors were used as biological replicates to account for inter-individual variability. To induce the NLPR3 inflammasome, human macrophages were exposed to LPS priming signal for 3.5 hours and activation signal ATP for 30 min post LPS stimulation. Control samples with only LPS or ATP were also generated to assay alongside. Confirmation of NLRP3 inflammasome activation and secretion of IL-1β was confirmed via ELISA (**Fig 5.1A**). For the non-canonical inflammasome, activation was achieved by exposing cells to the TLR ligand Pam3CSK4 for 4 hours, followed by overnight LPS transfection via lipofectamine 3000. As expected, this resulted in sufficient IL-1β secretion from LPS transfected samples but not from corresponding controls (**Fig 5.1B**). The canonical inflammasome was also stimulated in the presence of C75, a small molecule inhibitor of FASN, for 1 hour prior to ATP stimulation. Exposure of hMDMs to C75 resulted in a reduction in IL-1β secretion (**Fig 5.1C**). Lipid extraction was performed via a liquid-liquid extraction method, employing methanol and chloroform as solvents and water as the aqueous phase, followed by drying under vacuum (**Fig. 5.1D**).

The non-polar lipid layer of samples was then run using a kinetex C18 column, as described in the materials and methods section. Upon elution from the column, the samples were processed using a ThermoFisher Orbitrap Exploris 240, with an aliquot, pooled from the six samples, used for quality control assay. Collected data underwent centring to reduce bias, peak picking to isolate relevant signals, and normalisation using internal standards to correct for variability, ensuring accurate and comparable results across samples. Pre-processing of the samples was conducted via MS-Dial which also identified lipid classes (**Fig 5.1E**). Statistical analysis and visualisation were done using the open source MetaboAnalyst software. It should be noted that the MetaboAnalyst software does not allow modification of its predefined annotations, and thus colour schemes shown in the data generated remained inconsistent despite the efforts made.

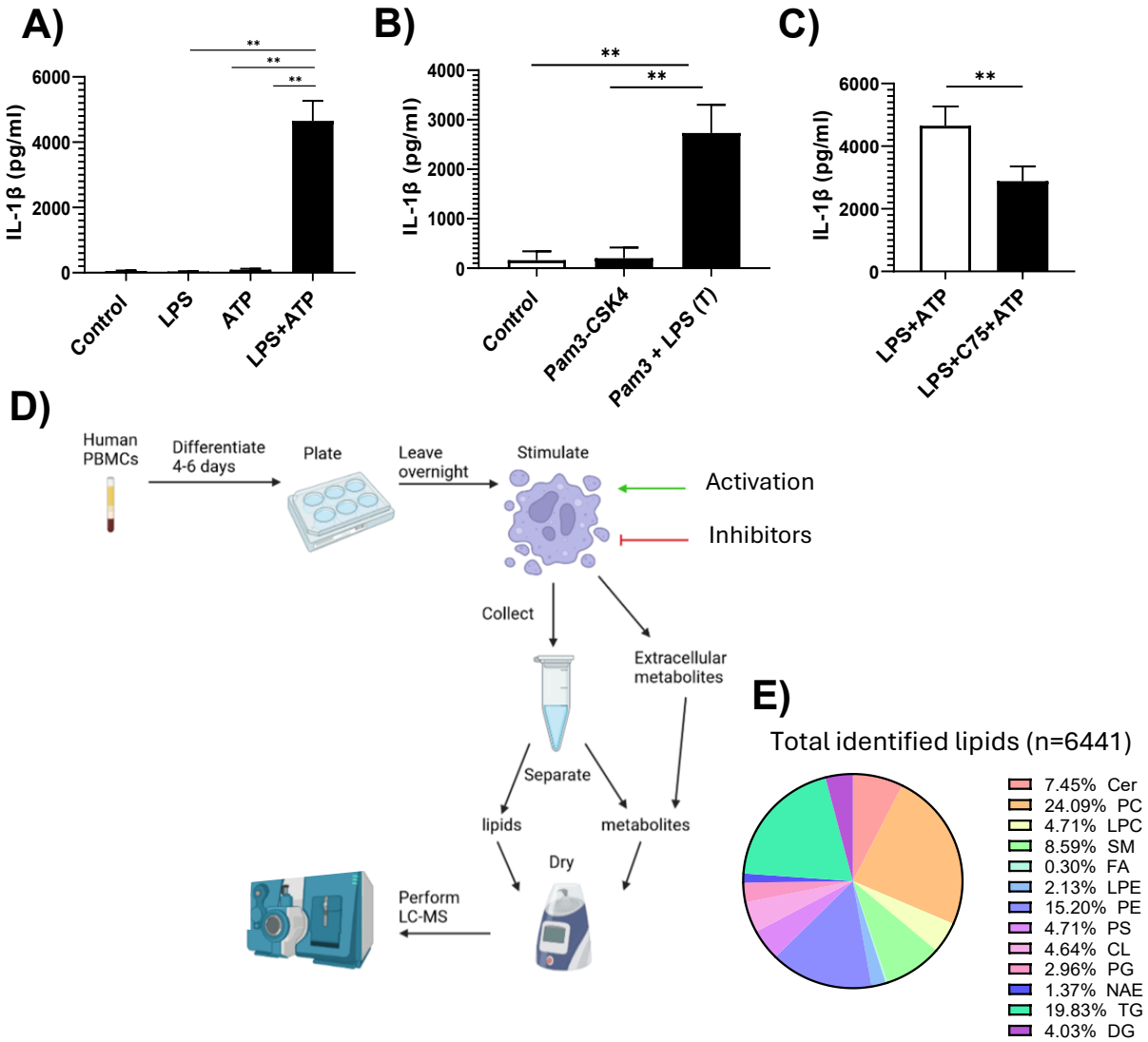


Figure 5.1 Overview of LC-MS workflow and confirmation of NLRP3 inflammasome activation. A) hBMDM were exposed to LPS(500ng/ml; 3.5h) or ATP (5mM 30min) or combination of LPS +ATP. **B)** hBMDM exposed to Pam3CSK4 (500 ng/mL; 4h) and Pam3CSK4 and transfected LPS (1ug/ml; overnight). **C)** PBMCs treated as in A but with exposure to C75 (12.5 μM; 2h). **D)** Overview of the LC-MS sample preparation**. E)** Breakdown of the total lipid class identified in the entire LC-MS run. Scale bars; $*p < 0.05$; $**p < 0.01$; by one-way ANOVA or students T test, n=6.

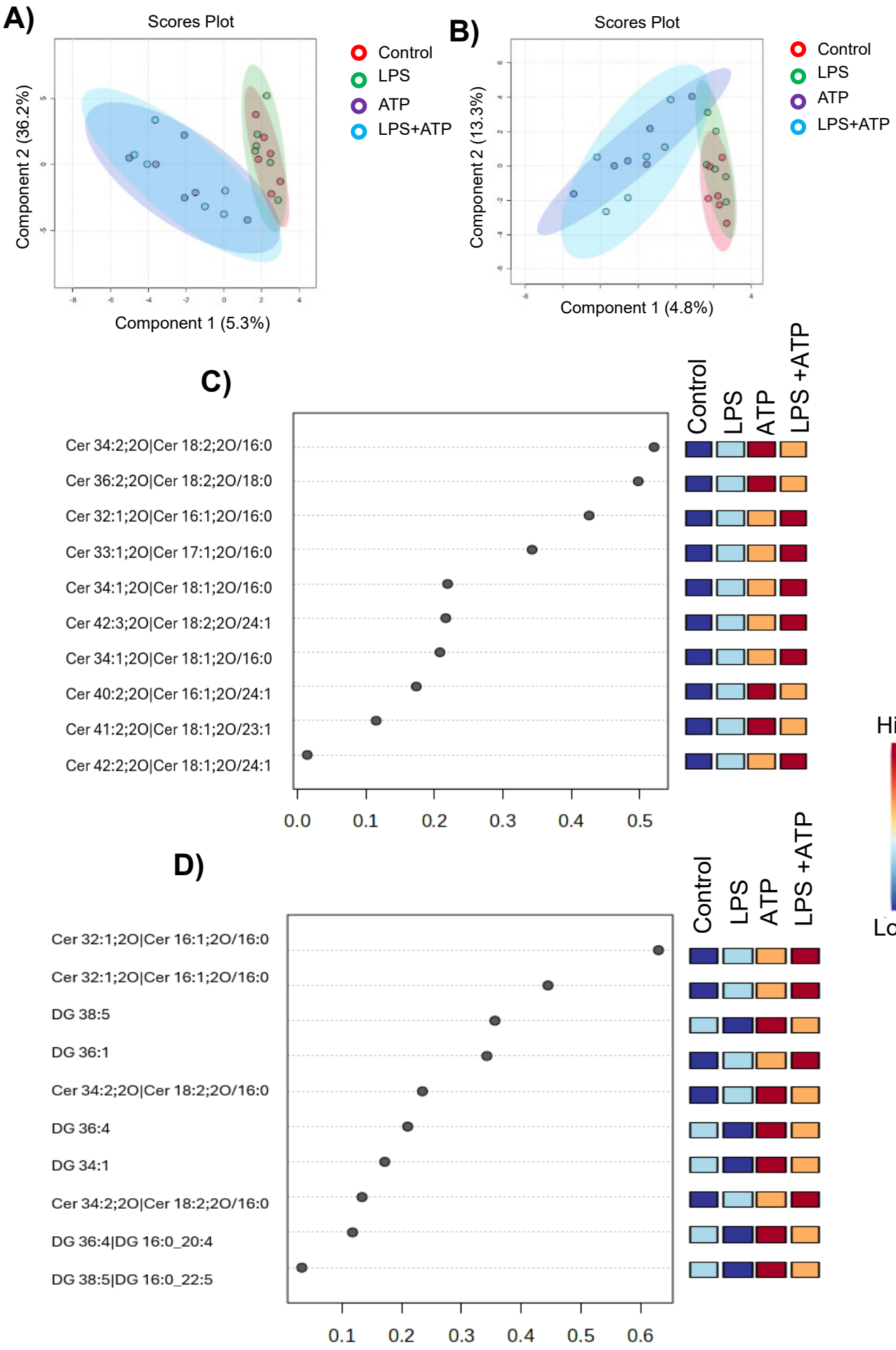
5.2.2 Activated NLRP3 induces a distinct lipidome primarily driven by ATP.

The individual effects of exposure to the NLRP3 priming signal LPS and activation signal ATP, along with the combined effect of LPS and ATP were initially compared. Partial Least Squares Discriminant Analysis (PLS-DA) is a multivariate statistical method used to find patterns in complex datasets by reducing dimensionality while maximising the separation between predefined groups. In this context, the PLS-DA analysis allowed for the differentiation of samples based on their distinct molecular profiles, highlighting the impact of LPS and ATP exposure on the samples. A shift in the PLS-DA was observed between the negative control and LPS-exposed samples, compared to those exposed to ATP and the combined LPS and ATP condition (**Fig. 5.2A, 5.2B**). The variable importance in projection (VIP) of lipids from the PLS-DA analysis was presented to characterise the broader lipidomic changes associated with NLRP3 inflammasome activation. VIP scores were derived from the PLS-DA models shown in Figures **5.2A** and **5.2B**, using the Statistically Inspired Modification of PLS (SIMPLS) algorithm within Metaboanalyst. These scores quantify the contribution of each lipid to the model's ability to distinguish between experimental groups. A higher VIP score indicates that a lipid has a greater influence on group separation in the PLS-DA plot. Therefore, these scores can be interpreted as identifying the key lipid species that drive the observed differences in lipidomic profiles between conditions. Among these, triglycerides, carnitine, and N-acylethanolamines (NAEs) were influenced by ATP treatment. The primary lipids driving alterations in the lipidome during inflammasome activation were identified as ceramides and diglycerides (DAG). Ceramides of varying carbon chain lengths contributed the most statistical weight in distinguishing the PLS-DA groups, underscoring their central role in these lipidomic shifts (**Fig. 5.2C, 5.2D**).

Previous studies have indicated that ceramide functions as a damage-associated molecular pattern (DAMP) in fat-induced obesity and plays a role in NLRP3 inflammasome activation by promoting mitochondrial dysfunction, oxidative stress, and alterations in cellular membrane integrity (Camell et al., 2015). The observed upregulation of ceramide in this study confirms NLRP3 inflammasome activation and demonstrates the ability of the mass spectrometry method to detect lipidomic changes. This serves as a control, lending credibility to subsequent findings.

Data were analysed in both positive and negative ionisation modes, allowing for a more detailed examination. The positive mode facilitates the detection of positively charged ions, while the negative mode captures more acidic lipids prone to deprotonation. By maintaining separation between the two modes, potential data obfuscation was avoided.

In addition to ceramide, significant lipidomic changes were attributed to diglycerides (DAG). Although DAGs have not been directly implicated as inducers of NLRP3 inflammasome activation, they may represent a regulatory pathway worthy of further investigation in the context of inflammasome research. Heatmap analysis revealed substantial shifts in the lipidome upon inflammasome activation, with ATP identified as the primary driver of these changes. A group of variable lipids, highlighted in the dotted outline, were identified as abundant: phosphatidylcholine (PC), phosphatidic acid (PA), and phosphatidylethanolamine (PE) (**Fig. 5.2E, 5.2F**). These phosphorylated lipids play critical roles in membrane integrity and cellular signalling, with PA functioning as a direct signalling molecule, and PE and PC acting as intermediaries in signalling pathways.


A)
Scores Plot
Component 2 (36.2%)
Component 1 (5.3%)
Control
LPS
ATP
LPS+ATP
B)
Scores Plot
Component 2 (13.3%)
Component 1 (4.8%)
Control
LPS
ATP
LPS+ATP
C)
Cer 34:2;2O|Cer 18:2;2O/16:0
Cer 36:2;2O|Cer 18:2;2O/18:0
Cer 32:1;2O|Cer 16:1;2O/16:0
Cer 33:1;2O|Cer 17:1;2O/16:0
Cer 34:1;2O|Cer 18:1;2O/16:0
Cer 42:3;2O|Cer 18:2;2O/24:1
Cer 34:1;2O|Cer 18:1;2O/16:0
Cer 40:2;2O|Cer 16:1;2O/24:1
Cer 41:2;2O|Cer 18:1;2O/23:1
Cer 42:2;2O|Cer 18:1;2O/24:1
0.0
0.1
0.2
0.3
0.4
0.5
Control
LPS
ATP
LPS +ATP
High
Low
D)
Cer 32:1;2O|Cer 16:1;2O/16:0
Cer 32:1;2O|Cer 16:1;2O/16:0
DG 38:5
DG 36:1
Cer 34:2;2O|Cer 18:2;2O/16:0
DG 36:4
DG 34:1
Cer 34:2;2O|Cer 18:2;2O/16:0
DG 36:4|DG 16:0_20:4
DG 38:5|DG 16:0_22:5
0.1
0.2
0.3
0.4
0.5
0.6
Control
LPS
ATP
LPS +ATP

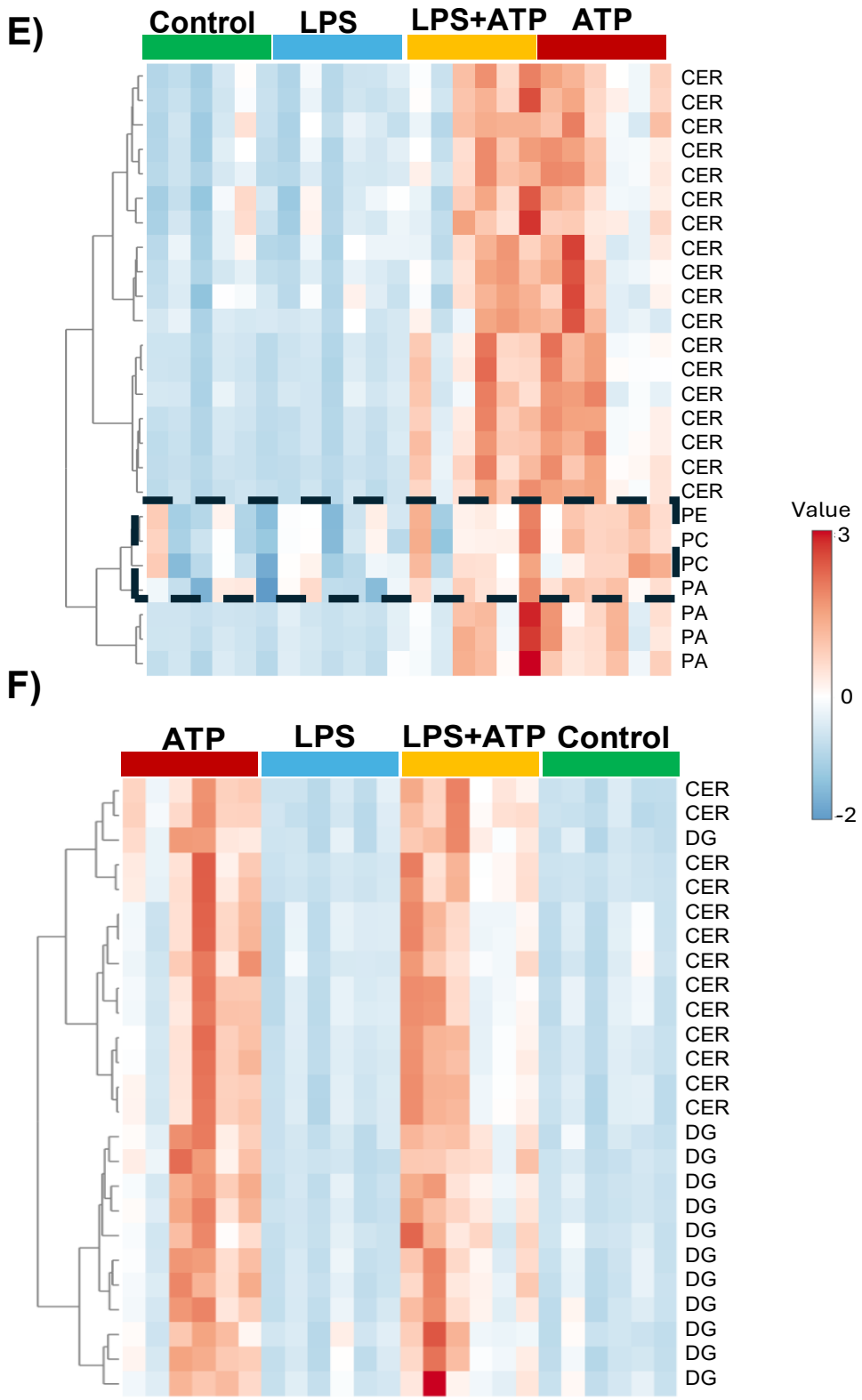
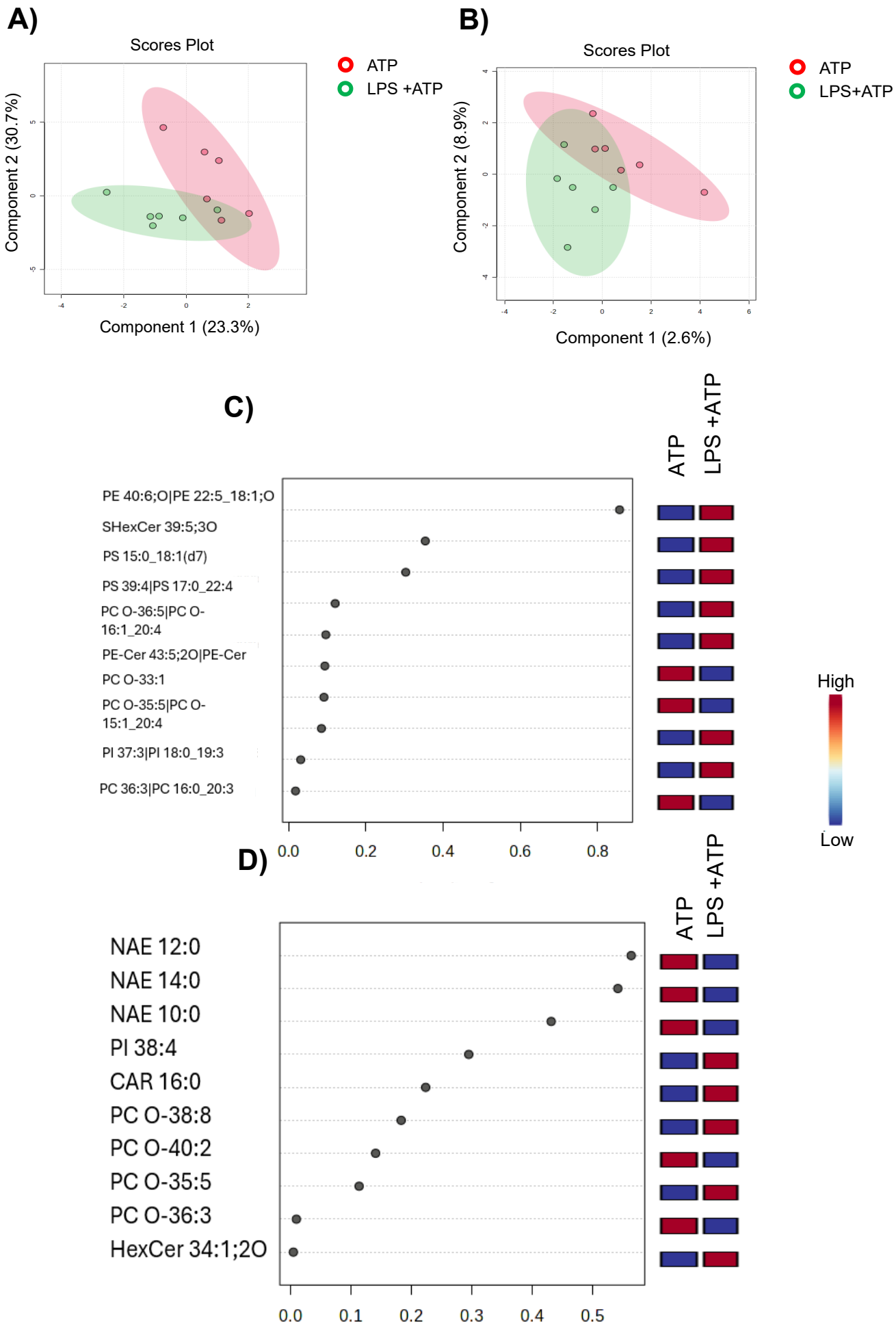


Figure 5.2: The activation signal of NLRP3 is the primary driver of shifts in the lipidome. A, B) PLS-DA of the negative fraction **(A)** and positive fraction **(B)** of human PBMCs treated with control, LPS (500 ng/mL; 3.5 h) and ATP (5 mM; 30 min), or a combination of both. **C, D)** VIP scores for the negative fraction **(C)** and positive fraction **(D)** of the PLS-DA shown in A and B. **E, F)** Heatmaps of the negative fraction **(E)** and positive fraction **(F)** for PBMCs treated as described in A and B. All MS experiments and analyses are based on six biological replicates per experimental condition. VIP scores are derived from the top 10 most significant lipids. Heatmap rows represent the top 25 most significantly altered lipids, as identified by one-way ANOVA. Z-scores were used to normalise lipid abundance, ensuring comparability of relative expression changes.

5.2.3 Examining the distinction between inflammasome activation

To further elucidate the lipid species driving changes in the lipidome during NLRP3 activation, a direct comparison was made between ATP-treated samples and those treated with both LPS and ATP. A notable shift between the two populations was observed in the PLS-DA analysis, indicating a sufficient separation to be considered significant by this method (**Fig. 5.3A, 5.3B**). However, it should be noted that the positive fraction exhibited a higher degree of similarity, with a lower percentage variance for both components, whereas the negative fraction demonstrated substantial variation, which was reflected in the lipid species identified.

A range of phosphorylated lipids, including PE, PS, and PC, was identified in the negative mode. These lipids were highly expressed in macrophages exposed to both LPS and ATP but not in macrophages treated with ATP alone, suggesting that the priming signal is critical for their regulation. Concurrently, a reduction in N-acylethanolamines (NAE) was observed (**Fig. 5.3C, 5.3D**). Heatmap analysis revealed that while ATP alone induces widespread lipidomic changes, only a selective subset of lipids, including PE, PS, and PC, is altered when the priming signal is also present (**Fig. 5.3E, 5.3F**). Changes in the levels of PE, PS, and PC may have significant implications for NLRP3 inflammasome activation and could contribute to the observed alterations in inflammasome activity.


A)
Scores Plot
ATP
LPS +ATP
Component 2 (30.7%)
Component 1 (23.3%)
B)
Scores Plot
ATP
LPS+ATP
Component 2 (8.9%)
Component 1 (2.6%)
C)
ATP
LPS +ATP
PE 40:6;O|PE 22:5_18:1;O
SHexCer 39:5;3O
PS 15:0_18:1(d7)
PS 39:4|PS 17:0_22:4
PC O-36:5|PC O-16:1_20:4
PE-Cer 43:5;2O|PE-Cer
PC O-33:1
PC O-35:5|PC O-15:1_20:4
PI 37:3|PI 18:0_19:3
PC 36:3|PC 16:0_20:3
0.0
0.2
0.4
0.6
0.8
High
Low
D)
ATP
LPS +ATP
NAE 12:0
NAE 14:0
NAE 10:0
PI 38:4
CAR 16:0
PC O-38:8
PC O-40:2
PC O-35:5
PC O-36:3
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0.0
0.1
0.2
0.3
0.4
0.5

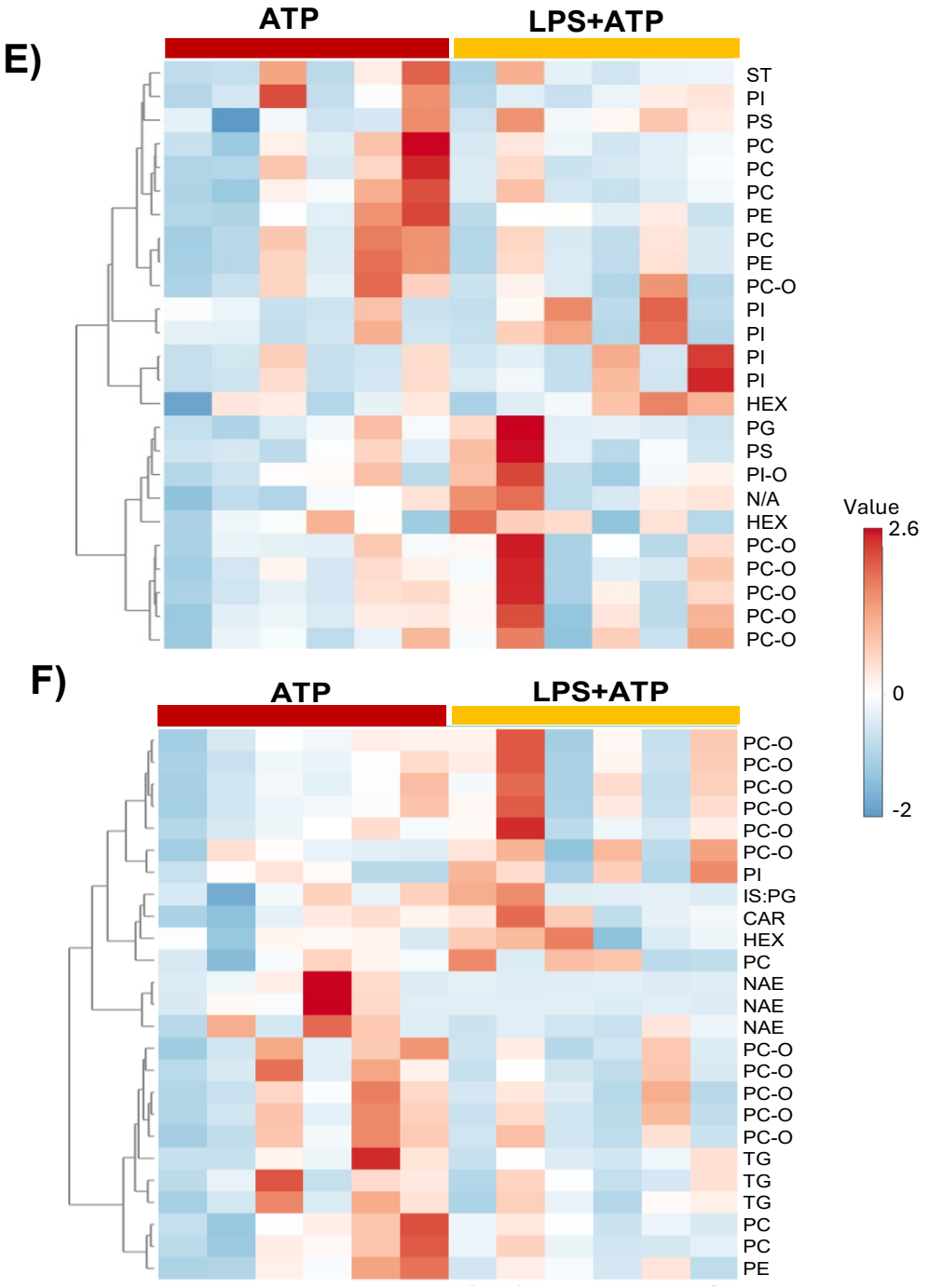
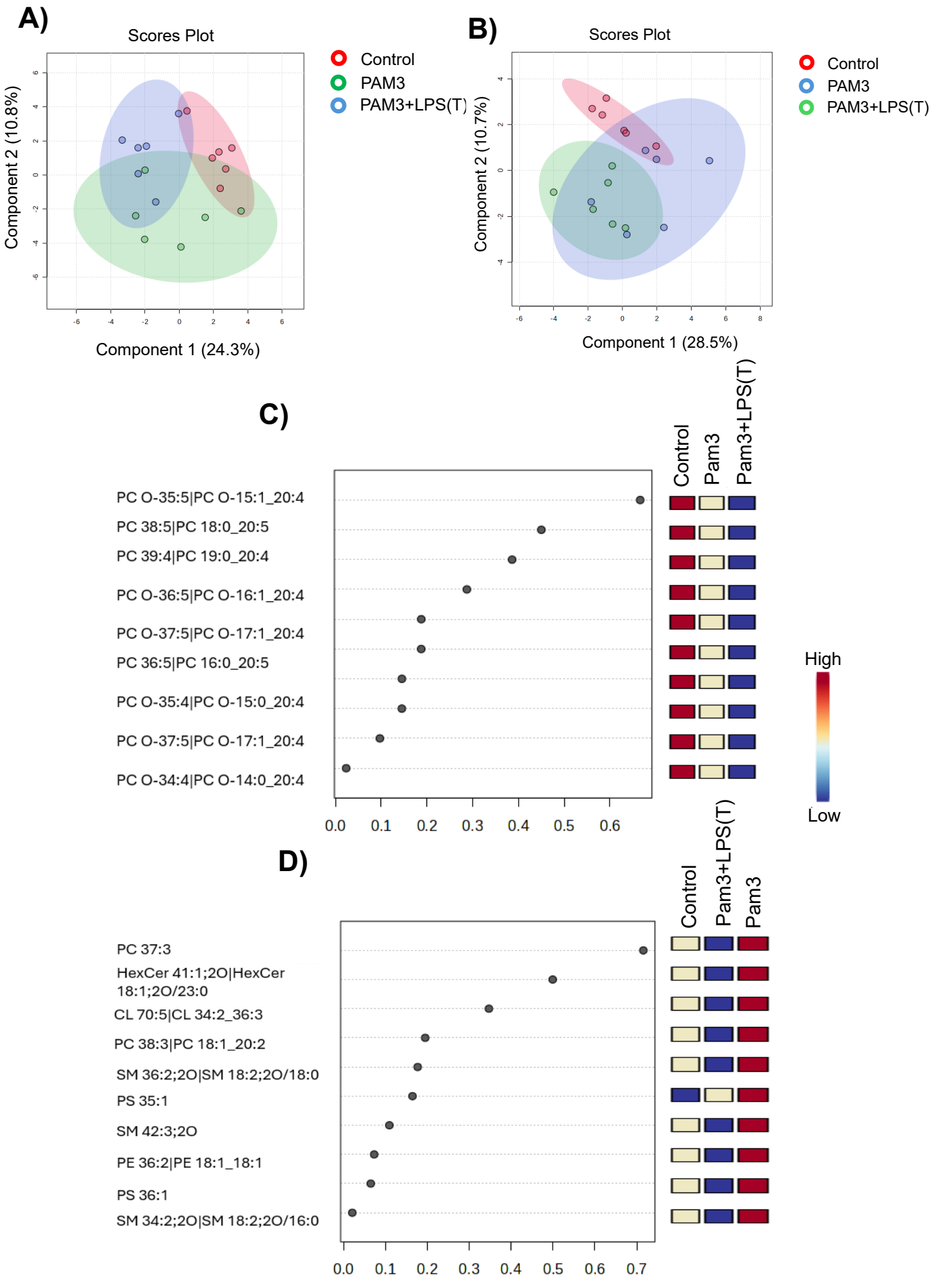


Figure 5.3: Activation signalling, in the presence of a priming signal, induces a shift in the lipidome. A, B) PLS-DA of the negative fraction **(A)** and positive fraction **(B)** of human PBMCs treated with control, ATP (5 mM; 30 min), or a combination of LPS (500 ng/mL; 3.5 h) and ATP. **C, D)** VIP scores for the negative fraction **(C)** and positive fraction **(D)** PLS-DA shown in A and B. **E, F)** Heatmaps of the negative fraction **(E)** and positive fraction **(F)** for macrophages treated as described in A and B. All MS experiments and analyses are based on six biological replicates per experimental condition. VIP scores are derived from the top 10 most significant lipids. Heatmap rows represent the top 25 most significantly altered lipids, as identified by Student's *t*-test. Z-scores were used to normalise lipid abundance, ensuring comparability of relative expression changes.

5.2.4 Changes in the lipidome of the non-canonical inflammasome

Following the elucidation of changes in lipids during the canonical inflammasome, the analysis was extended to investigate lipid remodelling during the non-canonical inflammasome. Human MDMs were exposed to TLR2 ligand Pam3CSK4, which is required to enhance caspase-11 expression for the activation of the non-canonical inflammasome. Following this, the cells were transfected with LPS to trigger caspase-11-dependent non-canonical inflammasome. Given that cell growth media may profoundly alter the external lipid environment, these experiments were conducted in reduced serum media which additionally enhanced transfection efficiency.

A clear distinction between the positive and negative lipid fractions was observed, with the positive fraction showing greater homogeneity between primed and activated cells (**Fig. 5.4A, 5.4B**). The scoring similarity within the positive fraction suggests a higher degree of lipid similarity compared to the negative fraction. Notably, the positive fraction contained a broader variety of lipids, with PC (37:3) emerging as a major contributor to the PLS-DA. This PC variant, characterised by fewer carbons and double bonds, may reflect a shift in the non-canonical inflammasome from long-chain to short-chain PC (**Fig. 5.4C, 5.4D**). Such a shift, characterised by shorter chains and fewer double bonds, is commonly associated with oxidative stress and inflammation (Leong, 2021; Guerbette *et al.*, 2024). Simultaneously, sphingomyelin (SM) lipids were found to be downregulated when LPS was transfected. Heatmap analysis further supported this observation, showing a lack of distinct clustering in the PLS-DA, which suggests a high degree of homogeneity between the tested conditions (**Fig. 5.4E, 5.4F**). These findings imply that while the overall lipid changes may be subtle, certain specific lipids could play a more pivotal role in influencing the non-canonical inflammasome's activation state, rather than the broader lipidomic environment.


A)
Scores Plot
Component 2 (10.8%)
Component 1 (24.3%)
Control
PAM3
PAM3+LPS(T)
B)
Scores Plot
Component 2 (10.7%)
Component 1 (28.5%)
Control
PAM3
PAM3+LPS(T)
C)
PC O-35:5|PC O-15:1_20:4
PC 38:5|PC 18:0_20:5
PC 39:4|PC 19:0_20:4
PC O-36:5|PC O-16:1_20:4
PC O-37:5|PC O-17:1_20:4
PC 36:5|PC 16:0_20:5
PC O-35:4|PC O-15:0_20:4
PC O-37:5|PC O-17:1_20:4
PC O-34:4|PC O-14:0_20:4
0.0 0.1 0.2 0.3 0.4 0.5 0.6
Control
Pam3
Pam3+LPS(T)
High
Low
D)
PC 37:3
HexCer 41:1;2O|HexCer 18:1;2O/23:0
CL 70:5|CL 34:2_36:3
PC 38:3|PC 18:1_20:2
SM 36:2;2O|SM 18:2;2O/18:0
PS 35:1
SM 42:3;2O
PE 36:2|PE 18:1_18:1
PS 36:1
SM 34:2;2O|SM 18:2;2O/16:0
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Control
Pam3+LPS(T)
Pam3

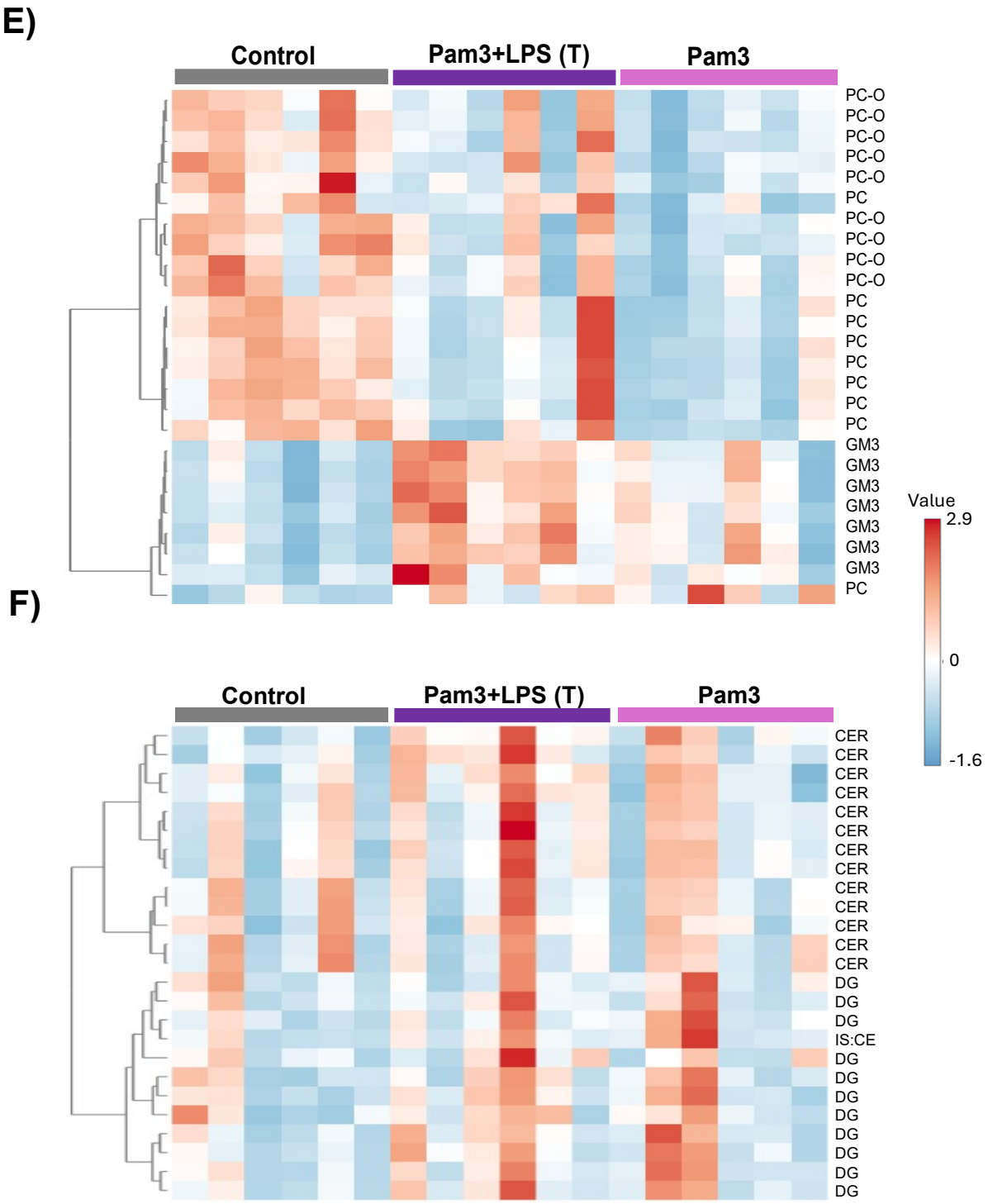


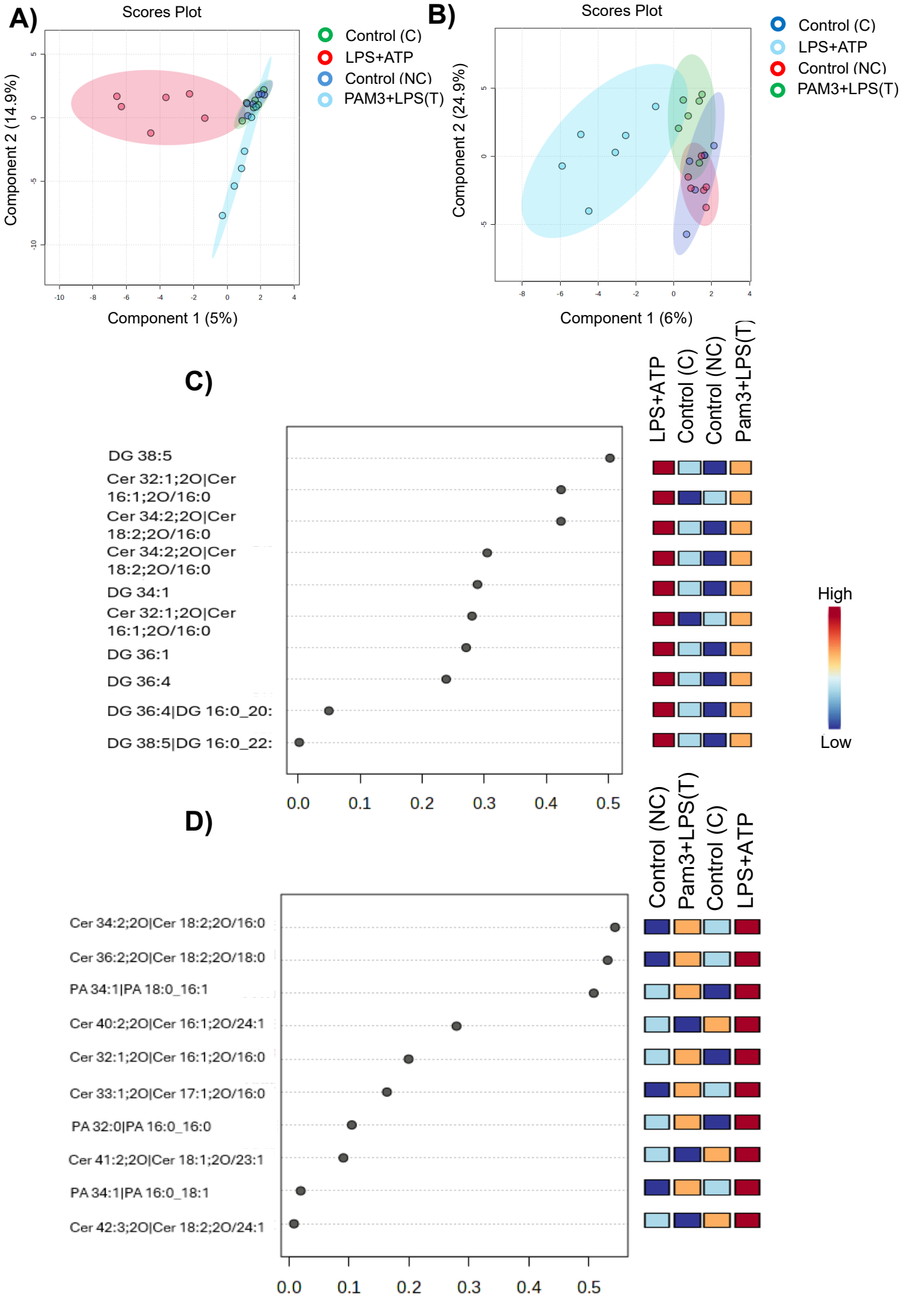
Figure 5.4: Reprogramming of the macrophage lipidome by non-canonical inflammasome. A, B) PLS-DA of the negative fraction **(A)** and positive fraction **(B)** of human PBMCs treated with control Opti-MEM, Pam3CSK4 (500 ng/mL; 4 hours), or Pam3CSK4 combined with transfected LPS (1 µg/mL; overnight). **C, D)** VIP scores for the negative fraction **(C)** and positive fraction **(D)** PLS-DA shown in A and B. **E, F)** Heatmaps for macrophages treated as described in A and B. All MS experiments and analyses are based on six biological replicates per experimental condition. VIP scores are derived from the ten most significant lipids. Heatmap rows represent the top 25 most significantly altered lipids, identified by one-way ANOVA. Z-scores were used to normalise lipid abundance, ensuring comparability of relative expression changes.

5.2.5 Analysis of the state of macrophage lipidome between canonical and non-canonical inflammasome

To explore the differences between the canonical and non-canonical inflammasomes upon activation, lipidome changes were examined and compared to their respective negative controls. PLS-DA revealed a clear separation between the lipid profiles of activated canonical and non-canonical inflammasomes compared to controls (**Fig. 5.5A, 5.5B**). Further analysis of the PLS-DA with VIP analysis revealed that DAG and ceramide and several PA lipids were significantly more abundant in both canonical and non-canonical inflammasomes compared to the negative controls (**Fig. 5.5C, 5.6D**). Heatmap analysis showed a global alteration in the lipidome, regardless of canonical or non-canonical activation (**Fig. 5.5E, 5.5F**). These results act as the backdrop for exploring the relationship between canonical and non-canonical inflammasome.

5.2.6 Identifying lipids that modulate the canonical and non-canonical inflammasome

Having confirmed a clear shift in the lipidome during NLRP3 activation compared to the respective negative controls, the analysis was extended to identify specific lipids distinguishing the canonical and non-canonical inflammasomes. This analysis focuses on identifying lipids that are uniquely influenced by each pathway. By comparing the two activation methods, we can isolate lipids specific to either the canonical or non-canonical inflammasome, eliminating those that are involved in both. The distinct lipid profiles for each activation method were revealed by PLS-DA, showing clear separation between the canonical and non-canonical inflammasomes (**Fig. 5.6A, 5.6B**). The primary lipid class significantly contributing to both the positive and negative fractions was identified as lysophosphatidylcholine (LPC). LPC has been reported as an active DAMP in the NLRP3 inflammasome, and its expression was exclusively observed in the canonical inflammasome (**Fig. 5.6C, 5.6D**) (Corrêa *et al.*, 2020). In contrast, the non-canonical inflammasome may rely on a more defined signalling pathway with fewer lipid mediators. Using heatmap analysis showed no large-scale differences between the canonical and non-canonical pathways, with both upregulated and downregulated lipids detected. However, within the negative fraction, a subpopulation of N-acylethanolamine (NAE) lipids, dotted outline, was observed. (**Fig. 5.6E, 5.6F**). While the distinction between canonical and non-canonical inflammasomes is less defined than that between activated and primed canonical NLRP3, these findings highlight several lipids for future investigation that could help clarify the differences between the two pathways.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Scores Plot
Component 2 (14.9%)
Component 1 (5%)
Control (C)
LPS+ATP
Control (NC)
PAM3+LPS(T)
B)
Scores Plot
Component 2 (24.9%)
Component 1 (6%)
Control (C)
LPS+ATP
Control (NC)
PAM3+LPS(T)
C)
DG 38:5
Cer 32:1;2O|Cer 16:1;2O/16:0
Cer 34:2;2O|Cer 18:2;2O/16:0
Cer 34:2;2O|Cer 18:2;2O/16:0
DG 34:1
Cer 32:1;2O|Cer 16:1;2O/16:0
DG 36:1
DG 36:4
DG 36:4|DG 16:0_20:
DG 38:5|DG 16:0_22:
0.0 0.1 0.2 0.3 0.4 0.5
LPS+ATP
Control (C)
Control (NC)
Pam3+LPS(T)
High
Low
D)
Cer 34:2;2O|Cer 18:2;2O/16:0
Cer 36:2;2O|Cer 18:2;2O/18:0
PA 34:1|PA 18:0_16:1
Cer 40:2;2O|Cer 16:1;2O/24:1
Cer 32:1;2O|Cer 16:1;2O/16:0
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PA 32:0|PA 16:0_16:0
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PA 34:1|PA 16:0_18:1
Cer 42:3;2O|Cer 18:2;2O/24:1
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Control (NC)
Pam3+LPS(T)
Control (C)
LPS+ATP

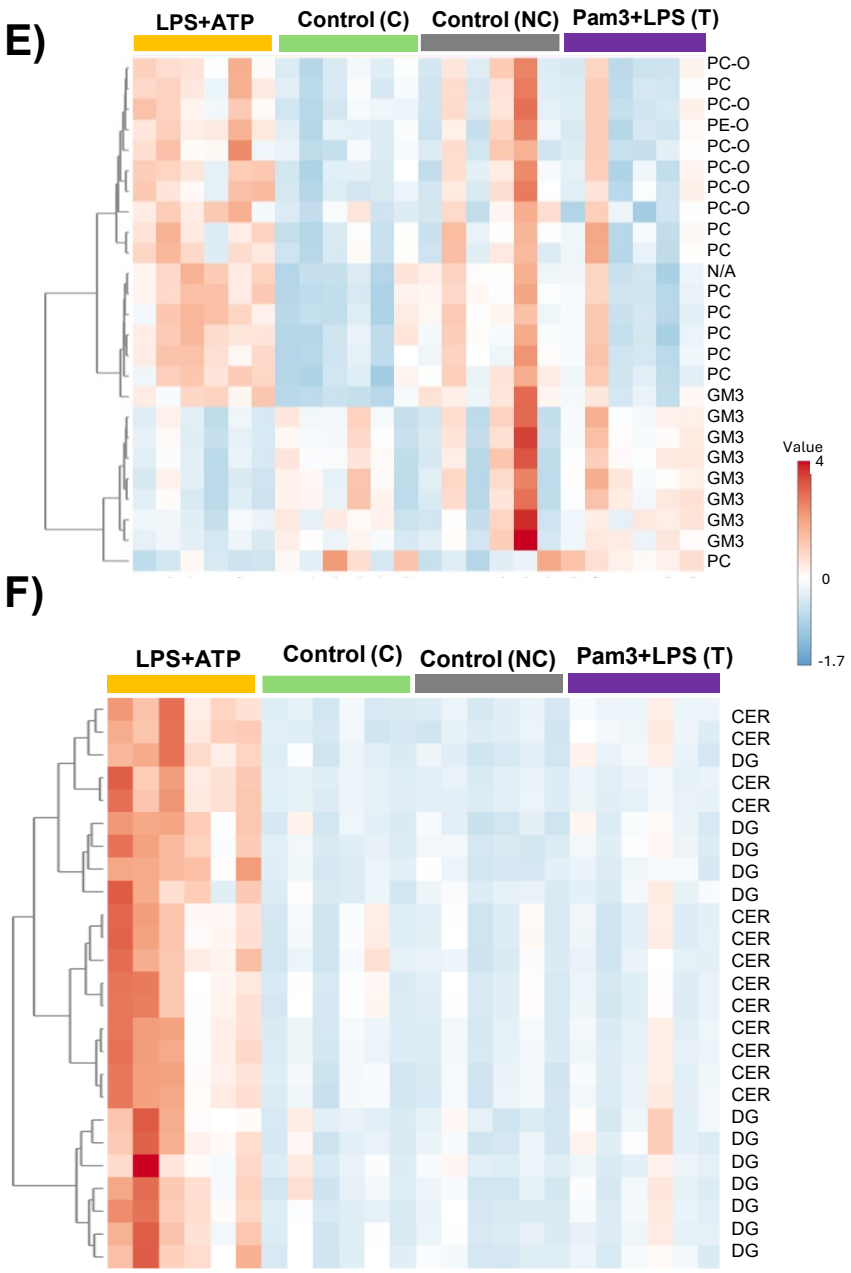
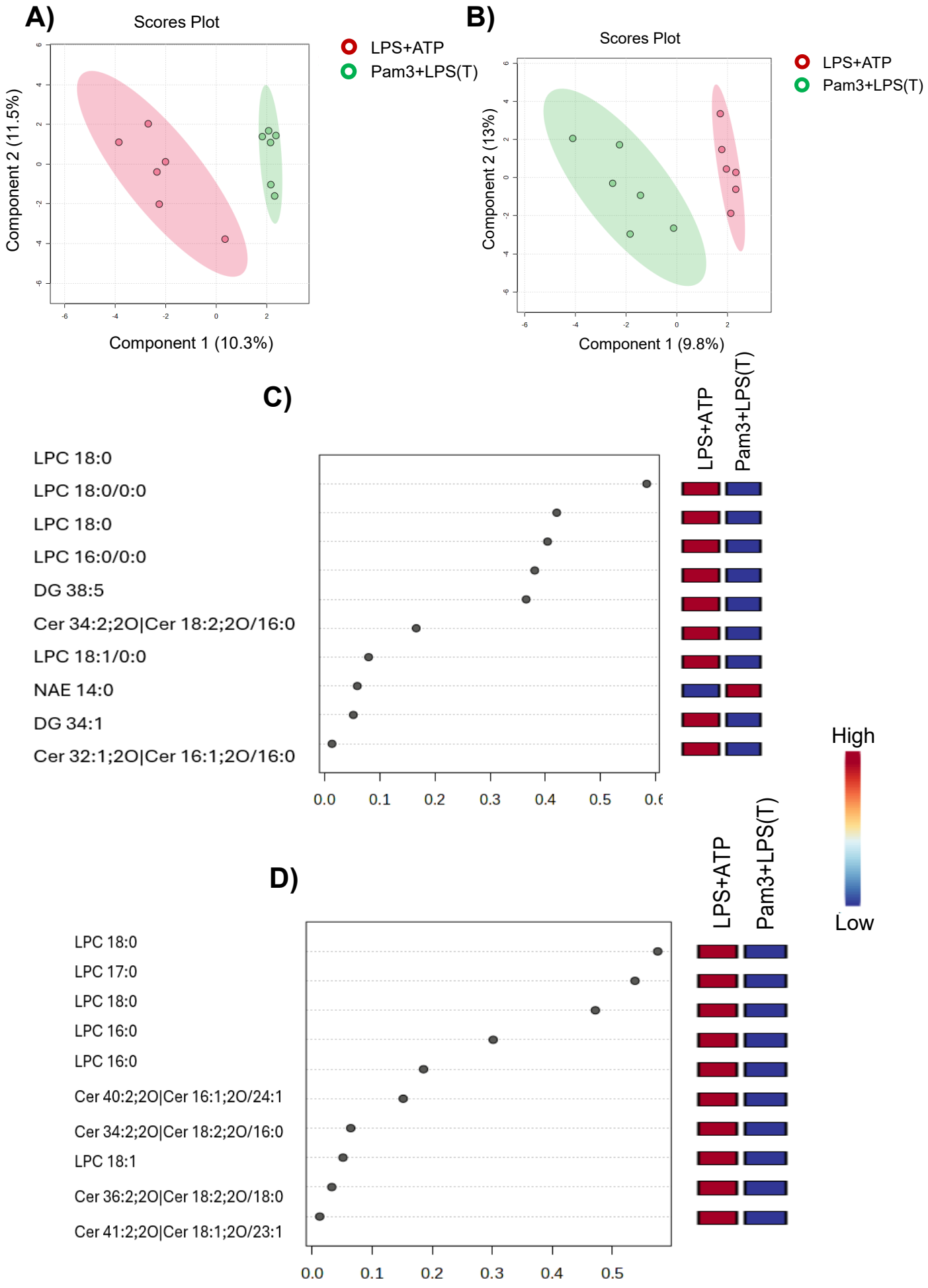


Figure 5.5: Canonical and non-canonical NLRP3 inflammasome produce distinct lipidomes. A, B) PLS-DA of the negative fraction **(A)** and positive fraction **(B)** of human PBMCs treated with either an active canonical inflammasome (LPS 500 ng/mL; 3.5 h, ATP 5 mM; 30 min) or a non-canonical inflammasome (Pam3CSK4 500 ng/mL; 4 hours and LPS 1 µg/mL overnight), and their respective controls. **C, D)** VIP scores for the negative fraction **(C)** and positive fraction **(D)** of the PLS-DA shown in A and B. **E, F)** Heatmaps of the negative fraction **(E)** and positive fraction **(F)** for macrophages activated as described in A and B. All MS experiments and analyses are based on six biological replicates per experimental condition. VIP scores are derived from the top 10 most significant lipids. Heatmap rows represent the top 25 most significantly altered lipids, as identified by one-way ANOVA. Z-scores were used to normalise lipid abundance, ensuring comparability of relative expression changes.


A)
Scores Plot
LPS+ATP
Pam3+LPS(T)
Component 2 (11.5%)
Component 1 (10.3%)
B)
Scores Plot
LPS+ATP
Pam3+LPS(T)
Component 2 (13%)
Component 1 (9.8%)
C)
LPC 18:0
LPC 18:0/0:0
LPC 18:0
LPC 16:0/0:0
DG 38:5
Cer 34:2;2O|Cer 18:2;2O/16:0
LPC 18:1/0:0
NAE 14:0
DG 34:1
Cer 32:1;2O|Cer 16:1;2O/16:0
LPS+ATP
Pam3+LPS(T)
High
Low
D)
LPC 18:0
LPC 17:0
LPC 18:0
LPC 16:0
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Cer 40:2;2O|Cer 16:1;2O/24:1
Cer 34:2;2O|Cer 18:2;2O/16:0
LPC 18:1
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Pam3+LPS(T)

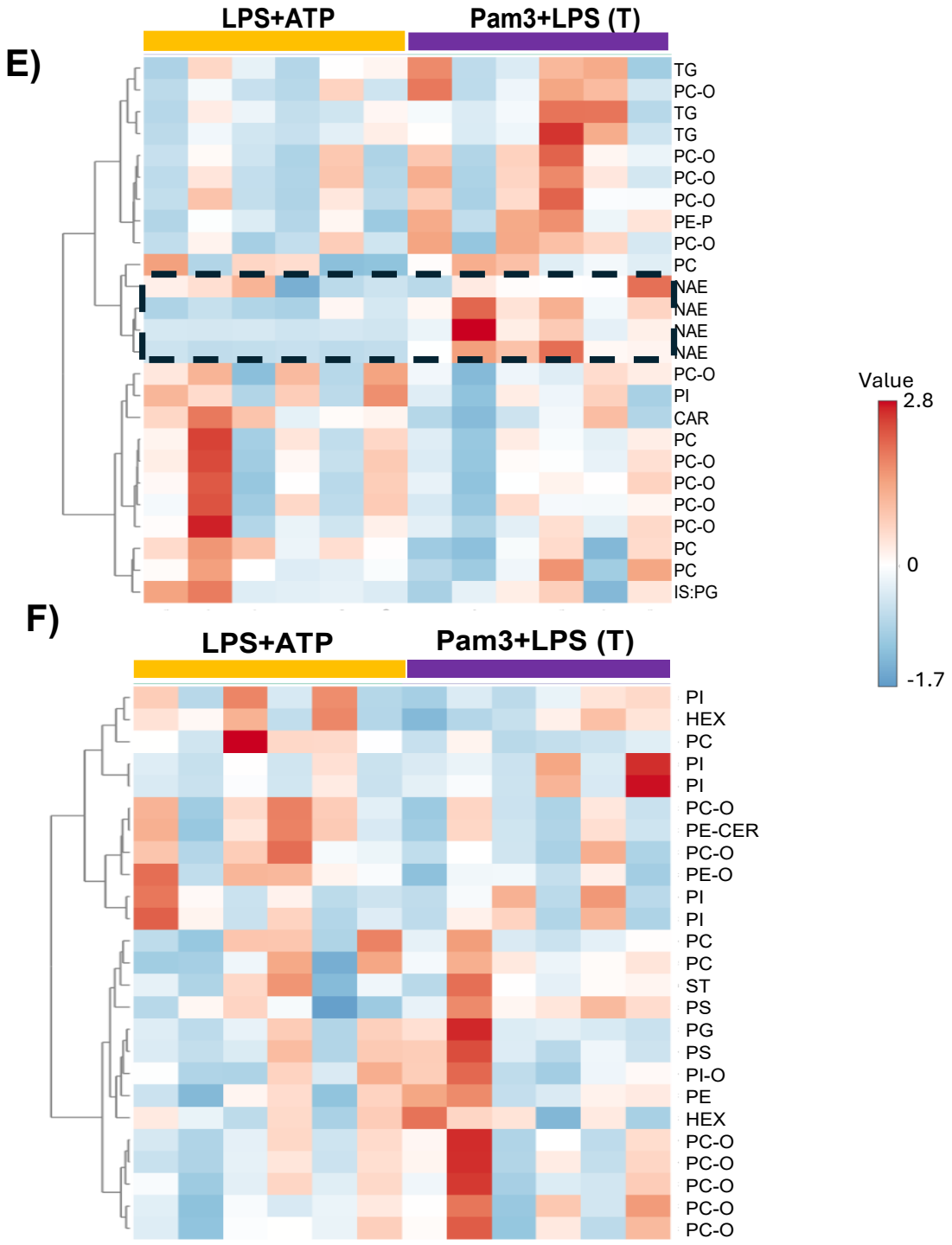
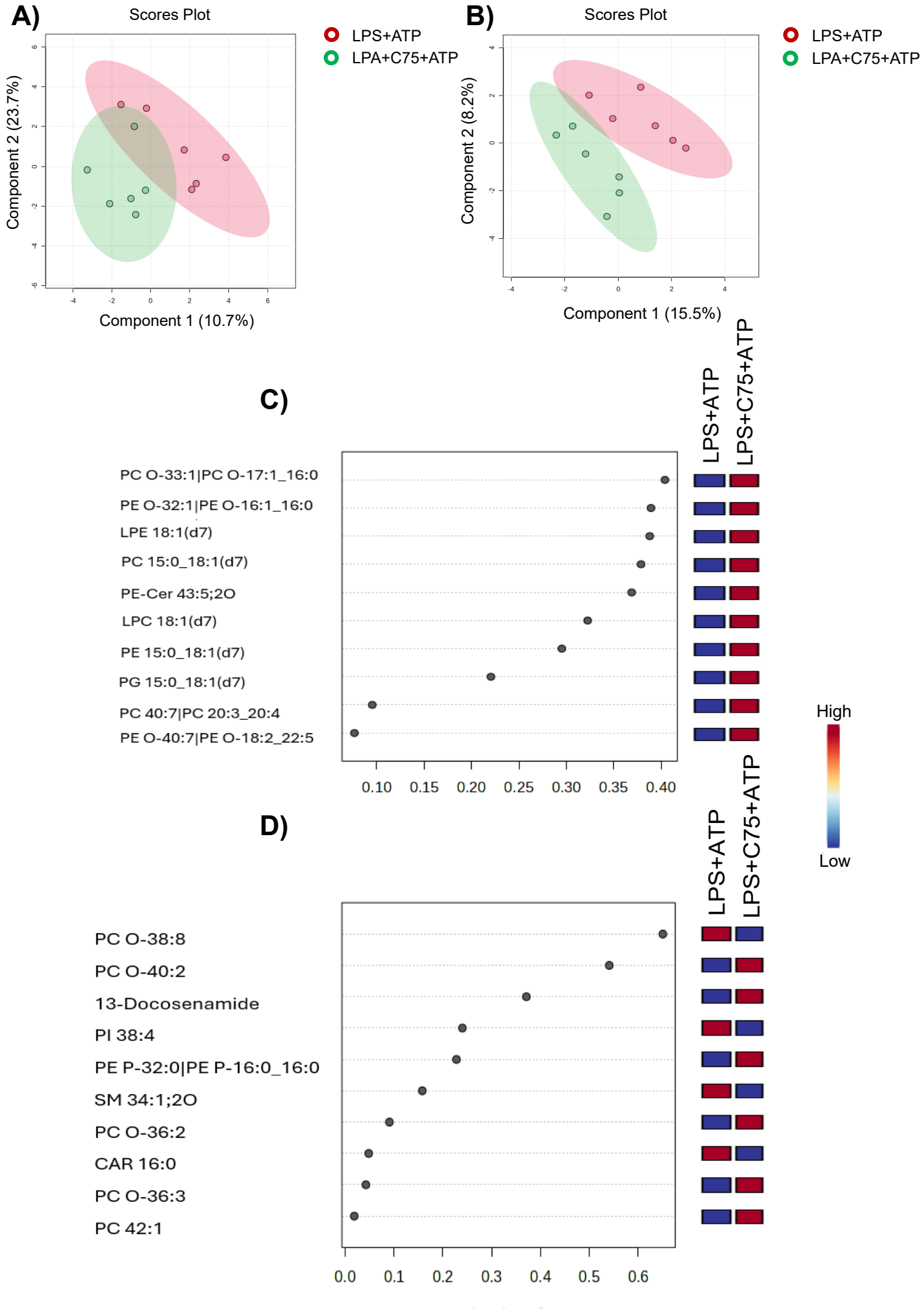


Figure 5.6: Direct identification of distinct lipidomes between canonical and non-canonical NLRP3 inflammasome. A, B) PLS-DA of the negative fraction **(A)** and positive fraction **(B)** of human PBMCs treated with either an active canonical inflammasome (LPS 500 ng/mL; 3.5 h, ATP 5 mM; 30 min) or a non-canonical inflammasome (Pam3CSK4 500 ng/mL; 4 hours and LPS 1 µg/mL overnight), and their respective controls. **C, D**) VIP scores for the negative fraction **(C)** and positive fraction **(D)** of the PLS-DA shown in A and B. **E, F)** Heatmaps of the negative fraction **(E)** and positive fraction **(F)** for macrophages activated as described in A and B. All MS experiments and analyses are based on six biological replicates per experimental condition. VIP scores are derived from the top 10 most significant lipids. Heatmap rows represent the top 25 most significantly altered lipids, as identified by one-way ANOVA. Z-scores were used to normalise lipid abundance, ensuring comparability of relative expression changes.

5.2.7 FASN influences the lipidome of canonical NLRP3 activated macrophages

To expand upon the preceding research in this thesis, the influence of fatty acid biosynthesis on the lipidome was examined. The canonical NLRP3 inflammasome was primed and activated in the presence or absence of the fatty acid synthesis inhibitor, C75. PLS-DA revealed a distinct population, as expected, indicating that fatty acid synthesis produces notable differences in the lipidome of macrophages (**Fig. 5.7A, 5.7B**). A diverse array of lipids, including phosphatidylcholine (PC), oxidised lysophosphatidylethanolamine (LPE), phosphatidylethanolamine (PE), ceramide, and phosphatidylglycerol (PG), were observed to be upregulated in the presence of C75 (**Fig 5.7C, 5.7D**). It can be speculated that the inhibition of fatty acid synthase (FASN) disrupts the overall lipid synthesis pathway, leading to the accumulation of specific key lipids at various stages of the pathway. Alternatively, these lipids may not be directly derived from FASN but could instead be influenced by inflammasome activation, persisting despite the inhibition of any new FASN-dependent fatty acid biosynthesis. Heatmap analysis indicated widespread population changes, likely attributed to a select few lipids exerting a significant influence on inflammasome activation (**Fig. 5.7E, 5.7F**). These findings suggest that fatty acid biosynthesis plays a critical role in shaping the lipidome during inflammasome activation and that specific lipids may serve as important mediators or markers in this process. Further investigation is needed to delineate the exact mechanisms through which fatty acid biosynthesis and lipid accumulation contribute to inflammasome modulation.


A)
Scores Plot
LPS+ATP
LPA+C75+ATP
Component 2 (23.7%)
Component 1 (10.7%)
B)
Scores Plot
LPS+ATP
LPA+C75+ATP
Component 2 (8.2%)
Component 1 (15.5%)
C)
PC O-33:1|PC O-17:1_16:0
PE O-32:1|PE O-16:1_16:0
LPE 18:1(d7)
PC 15:0_18:1(d7)
PE-Cer 43:5;2O
LPC 18:1(d7)
PE 15:0_18:1(d7)
PG 15:0_18:1(d7)
PC 40:7|PC 20:3_20:4
PE O-40:7|PE O-18:2_22:5
0.10 0.15 0.20 0.25 0.30 0.35 0.40
LPS+ATP
LPS+C75+ATP
High
Low
D)
PC O-38:8
PC O-40:2
13-Docosenamide
PI 38:4
PE P-32:0|PE P-16:0_16:0
SM 34:1;2O
PC O-36:2
CAR 16:0
PC O-36:3
PC 42:1
0.0 0.1 0.2 0.3 0.4 0.5 0.6
LPS+ATP
LPS+C75+ATP

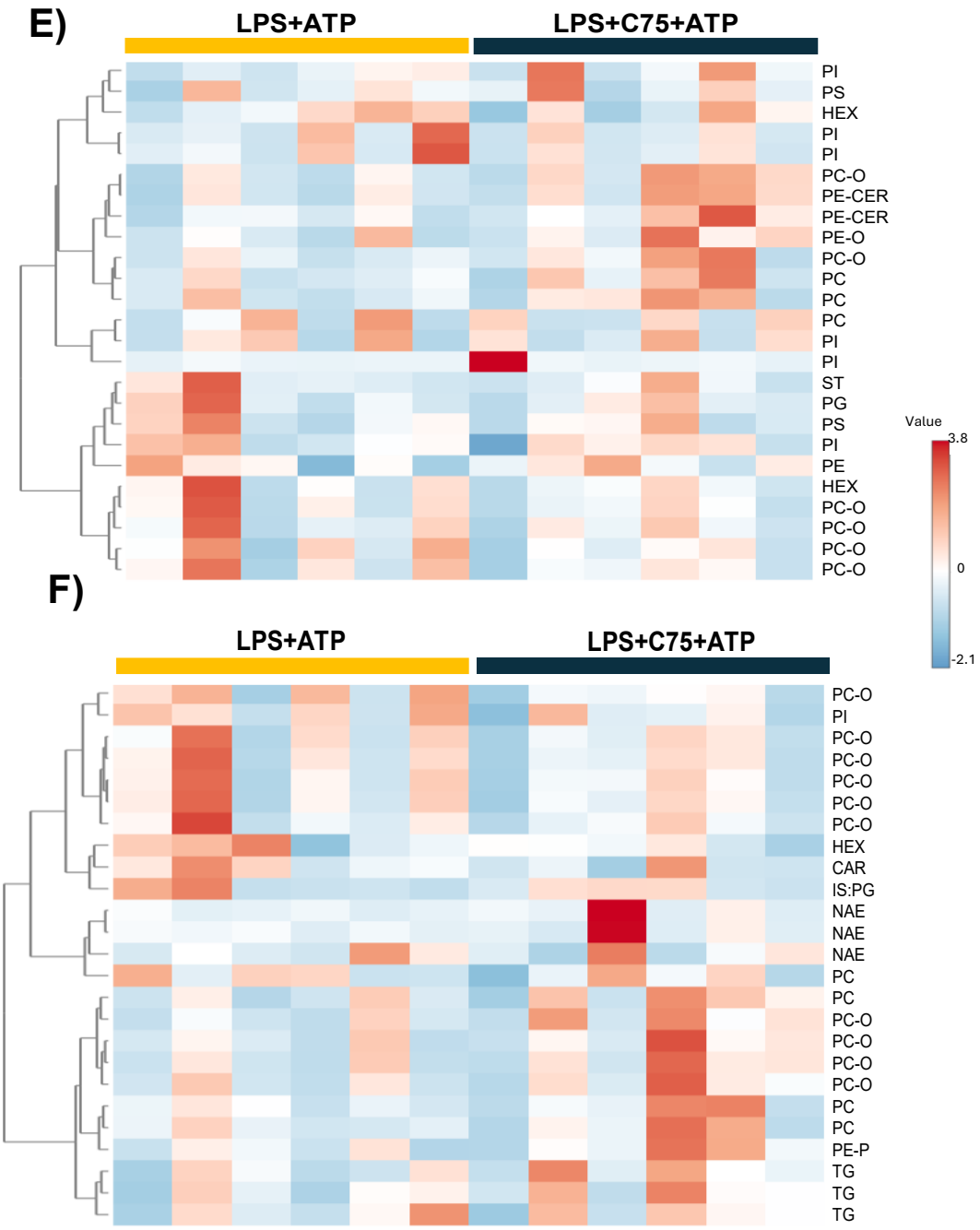


Figure 5.7: FASN produces a change in the lipidome of canonical NLRP3 activated macrophages. A, B) PLS-DA of the negative fraction (A) and positive fraction **(B)** of human PBMCs exposed to LPS (500 ng/mL; 3.5 h) and ATP (5 mM; 30 min), in the presence of C75 (12.5 µM; 2 h). **C, D)** VIP scores for the negative fraction **(C)** and positive fraction **(D)** from the PLS-DA shown in A and B. **E, F)** Heatmaps of the negative fraction **(E)** and positive fraction **(F)** for macrophages activated as described in A and B. All MS experiments and analyses are based on six biological replicates per experimental condition. VIP scores are derived from the 10 most significant lipids. The heatmap rows represent the 25 most significantly altered lipids, identified by T-test. Z-scores were used to normalise lipid abundance, ensuring comparability of relative expression changes.

5.3 Discussion

Within this chapter, both canonical and non-canonical pathways for NLRP3 inflammasome activation were explored. These distinct pathways were analysed using untargeted whole-cell LC-MS, with each pathway examined alongside its relevant controls. Comparisons between the pathways highlighted differences that might tailor the canonical and non-canonical inflammasomes to unique activation mechanisms. Building on previous chapters, the small molecule inhibitor C75 was employed alongside the activation of the canonical pathway. As a reminder, this inhibitor blocks the FASN pathway, resulting in profound effects on fatty acid synthesis. Notably, it also results in reduced palmitate production and subsequent NLRP3 palmitoylation. The use of C75 enabled further investigation into the broader lipid context during NLRP3 activation (Leishman *et al.*, 2024). This work provides an unbiased, comprehensive analysis of the lipidome during NLRP3 activation, taking a top-down approach rather than focusing on any specific pathway.

Significant shifts in the lipidome were observed in response to inflammasome priming and activation signals in macrophages, with the most pronounced changes occurring during inflammasome activation. The priming, LPS signal alone induced minimal lipid alterations compared to the negative control. Interestingly, the presence of ATP alone, regardless of priming, led to an increase in ceramide and diglyceride levels. This is notable, as previous studies suggest that ceramide regulates NLRP3 by stabilising the inflammasome complex (Li *et al.*, 2020). Once formed, ceramide localises to the plasma membrane, where its acyl chains interact to form stable lipid domains (Kolesnick *et al.*, 2000). These domains are associated with cellular reorganisation under stress (Hannun and Luberto, 2000). Beyond these structural roles, ceramide and its production by acid sphingomyelin have been reported to directly stimulate caspase-1 and IL-1β production in multiple studies (Bianco *et al.*, 2009; Grassmé *et al.*, 2014; Liu. *et al.*, 2022). Whilst the exact mechanism of ceramide activation of NLRP3 is unknown it is hypothesised to disrupt mitochondrial integrity leading to ROS generation (Liu. *et al.*, 2022). Alternatively, ceramide accumulation promotes clustering of the CD95 death receptor, triggering apoptosis. This process has also been linked to IL-1β secretion and maturation via caspase-8, independently of direct inflammasome activation (Grassmé *et al.*, 2001; Galli *et al.*, 2022). The findings presented in this chapter align with the hypothesis that ceramide not only modulates cellular reorganisation during NLRP3 activation but also facilitates this activation.

In cells exposed to ATP, another lipid class, diglyceride (DAG), was upregulated. While ceramide and DAG share structural similarities, they differ in function, which may lead to complementary roles in NLRP3 activation (Kolesnick *et al.*, 2000). While both ceramide and

DAG can form membrane domains, DAG tends to create more stable structures, whereas ceramide has been reported to induce membrane leakage (Artetxe *et al.*, 2017). The relationship between NLRP3 and DAG remains somewhat unclear, though there is some evidence of a predefined link. Increased DAG levels at the Golgi recruit and activate PKD, which then phosphorylates NLRP3, leading to its release from MAMs and the subsequent assembly of the fully mature inflammasome at the Golgi (Zhang *et al.*, 2017b). This pathway supports emerging evidence suggesting that the association of NLRP3 with the Golgi is essential for its activation, and this interaction is lipid-dependent (Zhang *et al.*, 2017a; Chen and Chen, 2018b). The findings presented are consistent with this previous research demonstrating that ATP induces upregulation of DAG resulting in the accumulation of lipids necessary for the full inflammasome activity. This hypothesis could be further tested through selective knockdown of DAG production pathways or via lipid analogues to introduce controls for DAG expression.

As LPS serves as the priming signal, its combination with ATP leads to full inflammasome activation, resulting in membrane rupture and pyroptosis. By restricting the comparison to these two conditions, it was possible to directly identify lipids most strongly associated with the transition from a primed to an activated state. While this focused approach highlights key drivers of activation, it also lays the groundwork for future studies to explore broader lipidomic changes across additional timepoints, doses, or inflammasome pathways. Notably, it was observed that there was a downregulation of NAE in LPS and ATP condition compared to just ATP alone. NAE are long-chain polyunsaturated fatty acids produced via a non-oxidative pathway, facilitated by N-acyltransferases (Blancaflor *et al.*, 2014). NAE lipid classes has been studied for its anti-inflammatory properties, with evidence suggesting it reduces the production of pro-inflammatory cytokines by suppressing NLRP3 activation (Tyrtyshnaia *et al.*, 2022). The inhibitory potential of NAE was reflected in the findings, where full inflammasome activation corresponded with a reduction in NAE. This suggests a loss of this regulatory lipid may be required upon NLRP3 activation.

A comparison between the NLRP3 canonical and non-canonical pathways revealed increased abundance in several distinct lipid classes. Typical lipid alterations associated with inflammasome activation were initially detected, but a notable difference was found in the levels of LPC, which was elevated in the canonical pathway but not in the non-canonical pathway. LPC has been associated with the activation of the NLRP3 inflammasome in foam cells during atherosclerosis and is implicated in membrane pore formation during pyroptosis (Corrêa *et al.*, 2020). The fact that LPC levels were not increased in non-canonical pathway-treated macrophages suggests that LPC may not be as crucial to pore formation in the non-

canonical pathway. However, without further investigation, the precise roles of LPC during canonical and non-canonical inflammasome activation remain unclear.

Rather than a single predominant lipid species distinguishing the effect of FASN inhibition, a diverse array of lipid changes was observed in macrophages treated with C75. These include phosphatidylcholine, oxidised lysophosphatidylethanolamine, phosphatidylethanolamine, ceramide, and phosphatidylglycerol. While these lipids originate from different pathways, they share a common relationship with membrane structure and integrity (Cullis and De Kruijff, 1979; Shimizu, 2009; Vance and Tasseva, 2013; Kolczynska *et al.*, 2020; Morris and Mitchell, 2023). Phosphatidylcholine, the most abundant phospholipid in mammalian membranes, maintains bilayer structure and regulates fluidity and permeability (van der Veen *et al.*, 2017). Phosphatidylethanolamine, with its cone-shaped structure, influences membrane curvature as well as being essential to inner mitochondrial membranes (Epand, Fuller and Rand, 1996; Joshi *et al.*, 2012). Oxidised lysophosphatidylethanolamine remodels membranes during oxidative stress, affecting fluidity and signalling (Petan and Manček-Keber, 2022). Phosphatidylglycerol, enriched in bacterial and mitochondrial membranes, enhances stability, protein function, and serves as a cardiolipin precursor (Morita and Terada, 2015). The collective contribution of these lipids underscores the importance of lipid homeostasis in maintaining membrane function and integrity, particularly under altered metabolic conditions induced by FASN inhibition.

It should be noted that in experiments using C75 in human BMDMs, IL-1β ELISA results showed only ~35–40% inhibition, compared to the near-complete (~100%) inhibition observed in earlier chapters. This discrepancy may stem from differences in cell type, as previous experiments were conducted in murine pBMDM. Species-specific variation in inflammasome regulation, FASN dependency, or sensitivity to pharmacological inhibitors may explain this outcome. Interestingly, while murine cells displayed reduced inhibition of cleaved caspase-1 in HEK293T overexpression systems (Chapter 4), human BMDMs now appear more resistant to FASN inhibition by C75. This may reflect differences in FASN regulation between species or differences in drug response between primary cells and immortalised lines.

In recent years, the lipidome has emerged as a critical focus in understanding health and disease (Dueñas *et al.*, 2023), with significant advancements towards maintaining an optimised lipid profile, particularly regarding the role of lipids in immune regulation (Stella Ademowo, Wenk and Maier, 2024). The expanding body of research on metabolic disorders, alongside advancements in lipid analysis technologies, has offered unprecedented insights into the lipidome's response to large-scale cellular alterations (Salihovic *et al.*, 2023). Whilst there is a variety of lipids which are predominantly upregulated in cells treated with C75, there

are select number of lipids which are more pronounced in activated NLRP3 macrophages – SM, Car and PC-O (38:8). The 'O' indicates that PC contains an ether bond, which imparts distinct properties compared to regular PC. PC-O (38:8) is of particular interest as in C75 treated cells PC-O (40:2) is more abundant, suggesting a shift from highly polyunsaturated to the less saturated, which could be due to fatty acid desaturation or oxidative stress (Assies *et al.*, 2014).

Given the differences observed between canonical and non-canonical activation, further exploration should focus on how specific lipid changes functionally impact the two pathways. For instance, it remains unclear whether certain lipids like LPC or NAE are more critical in modulating one pathway over the other. This could be tested through the addition of lipid inhibitors or mimetics (Stutz *et al.*, 2017; Majeed *et al.*, 2021). To build on these findings, the NLRP3-specific lipids identified in this study could be quantified using LC-MS/MS triple quadrupole mass spectrometry combined with multiple reaction monitoring (MRM). This approach enables the precise quantification of known lipids and allows researchers to determine how NLRP3 activation directly alters their levels. MRM, which involves tracking pre-selected lipids across various time points, could provide valuable insight into the specific transitions and dynamic changes in lipid composition during NLRP3 activation (Saleem, Mueller and Bhattacharya, 2023). Finally, it would be pertinent to examine the lipids observed within disease models relevant to metabolic disorders, such as obesity-induced inflammation or atherosclerosis (Vandanmagsar *et al.*, 2011; Swanson, Deng and Ting, 2019).

Certain limitations should be noted when reviewing this data. The untargeted nature of the method precluded precise quantification of individual lipid changes, restricting the analysis to shifts in lipid classes and their statistical significance relative to other detected lipids (Cajka and Fiehn, 2014). Additionally, limitations in assessing the state of inflammasome activation were noted. The observed changes may be present in only a subset of macrophages, as inflammasome activation is unlikely to occur uniformly across the entire macrophage population (He, Hara and Núñez, 2016; Qian *et al.*, 2019). Furthermore, the extent of mitochondrial damage induced by ATP may result in differential rates of inflammasome activation among macrophages (Elliott and Sutterwala, 2015). A significant challenge in lipidomic analysis lies in establishing causality; the lipidome is a dynamic and complex network, making it difficult to determine whether the lipids identified as influencing inflammasome activation are directly involved in its regulation or are merely responsive to upstream signals (Wenk, 2005). Whilst there are some limitations, these should not detract from the wealth of information offered by this analysis.

Lipidome analysis provides valuable insights into the mechanisms of inflammasome activation by identifying lipid species that may act as regulators or signalling molecules in this process. The long-term goal is to uncover lipid signatures that consistently correlate with inflammasome activation, potentially revealing novel therapeutic targets or biomarkers for inflammatory diseases. Our findings suggest that specific alterations in lipid classes, such as glycerophospholipids and sphingolipids, may influence inflammasome signalling, likely through effects on membrane dynamics or secondary messenger generation. These results will guide follow-on studies aimed at functionally validating key lipid species using genetic or pharmacological tools, and integrating lipidomics with transcriptomic or proteomic data to better understand the metabolic-immune interface. Within this chapter the cellular content of NLRP3 inflammasome activation has been assessed, revealing the most abundant lipid classes associated with the inflammasome activation. By providing this insight, it was established which lipids could potentially regulate the NLRP3 inflammasome during activation, and the transition from priming to activation. This work builds a platform for future research into the NLRP3 inflammasome relationship with the lipidome.

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Chapter 6

Summary and future perspectives

Chapter 6. Summary and future perspectives

6.1 Fatty acid synthesis and the NLRP3 inflammasome

NLRP3 inflammasome assembly can be influenced by a wide range of factors. With the signals that trigger activation, the specific proteins involved, their localization, and the pre-activation licensing all significantly influencing inflammasome assembly (Schroder and Tschopp, 2010; Guo *et al.*, 2018). Moreover, it is becoming increasingly clear that cellular context plays a critical role in inflammasome activation. Factors such as lipid availability and abundance can impact when the inflammasome forms (Listenberger *et al.*, 2003; Florance and Ramasubbu, 2023; Zhang. *et al.*, 2023). These insights are further highlighted by studies on the steps before the inflammasome complex assembles. These prior steps have revealed that the spatial localisation of NLRP3 is essential for its activation and consequently influences the process by which oligomerisation occurs.

This dissertation establishes a relationship between the NLRP3 inflammasome activation and FASN. It was demonstrated that the acute inhibition of FASN-mediated palmitate synthesis led to a marked reduction in key markers of NLRP3 activation, including cleaved caspase-1, GSDMD, and the secretion of mature IL-1β. Importantly, this relationship was specific to NLRP3 and did not extend to other inflammasomes, such as AIM2 and NLRC4. The observed inhibition builds upon previous studies that linked NLRP3 to cholesterol biosynthesis via FASN. In these studies, the production of acetoacetyl-ACP by the fatty acid synthesis pathway mediated priming of the NLRP3 inflammasome through the formation of lipid rafts (Wei *et al.*, 2016; Carroll *et al.*, 2018). The research contained here proposes that other FASN enzymatic sites, including β-ketoacyl-ACP synthase, β-ketoacyl-ACP reductase and thioesters, are additionally crucial for NLRP3 inflammasome activation(Kridel *et al.*, 2004; Hardwicke *et al.*, 2014). Given that the primary product of FASN is palmitate, it follows that the inability of FASN pathway to synthesize palmitate directly impacts NLRP3 palmitoylation and activation.

When palmitate conjugated to BSA was exogenously supplemented to macrophages, NLRP3 activation was restored despite FASN inhibition. This finding provides further evidence that palmitate is required for functional NLRP3 inflammasome. While previous studies have associated palmitate to the NLRP3 inflammasome, the precise nature of their relationship wasn't fully elucidated. As a saturated fatty acid, palmitate uptake induces changes in the cellular environment, potentially leading to lysosomal damage through crystallisation, leading to cathepsin release or the production of mtROS (Wen *et al.*, 2011b; Karasawa *et al.*, 2018a). The relationship between palmitate and NLRP3 was predominantly explored using the palmitate analogue inhibitor 2BP. Additionally, mutagenesis studies revealed that NLRP3 activation requires palmitoylation specifically at the Cys898 amino acid located within the LRR

domain of NLRP3. This builds on previous work which underscores the NLRP3 spatial localisation to the dTGN and association with PI4P. Currently, this an area of intense discussion with other studies corroborating these results. However, distinct NLRP3 residues that undergo palmitoylation have been reported with palmitoylation at Cys838 demonstrated to promote inflammasome activation (Zheng *et al.*, 2023). At the same time, NLRP3 Cys844 palmitoylation has been suggested to prevent extended inflammasome activation, with animals lacking the palmitoylacyltransferase exhibiting increased severity during an endotoxin shock model (Wang *et al.*, 2023). This contrasting relationship between different sites of palmitoylation mirrors other PTMs such as phosphorylation and ubiquitination. It is plausible that distinct NLRP3 cysteine sites are palmitoylated at different times within the NLRP3 life cycle, allowing for overall induction or inhibition of NLRP3 inflammasome depending on the strength or duration of the initial priming and activation signals.

While this research emphasises the importance of FASN-dependent NLRP3 Cys898 palmitoylation in activating the inflammasome, it acknowledges a limitation: the specific mechanism by which Cys898 controls NLRP3 activation is yet to be fully understood. What has been established, however, is that Cys898, located within the NLRP3 polybasic region, interacts with the TGN through a PI4P-mediated binding mechanism. As other studies have shown, the addition of palmitate facilitates membrane anchoring, such as in the case of Ras and heterodimeric G proteins where it was demonstrated that on occasion multiple anchors are required for membrane attachment (Hancock, Paterson and Marshall, 1990; Morales *et al.*, 1998). It is feasible that NLRP3 would similarly require additional different anchors to allow it to bind to the dTGN. This work did not identify the precise relationship between NLRP3 and its palmitoylation site beyond its association with the dTGN. As other PTMs can induce conformational changes within their target protein, it is possible that the Cys898 is able to change NLRP3 structure significantly. This hypothesis could be explored via cryo-electron microscopy, which would allow for the NLRP3 structure with the Cys898 mutation to be structurally characterised. Furthermore, as the Cys898 mutation studied in this work is located within the LRR domain, the hypothesised site of NLRP3 signalling activation, the introduction of this mutation could further limit the ability of NLRP3 to respond to its stimuli (Niu *et al.*, 2021). This could be further explored by testing a broader range of signals, along with binding interaction studies, though the theory that NLRP3 does not directly bind to its stimuli would make direct examination of its binding to the signal challenging.

While some of the other studies demonstrating NLRP3 palmitoylation have successfully identified the upstream acyltransferase involved, the enzyme responsible for palmitoylation at the Cys898 site was not explored in the current research. Future studies examining the acyltransferase could reveal how Cys898 is maintained and whether different cellular contexts

affect the dynamics of its palmitoylation. Intriguingly, by systematically examining the distinct functions of NLRP3 priming and activation signals, this research concluded that inflammasome priming, rather than the activation stimulus, mediates NLRP3 Cys898 palmitoylation. This was overlooked in other complementary studies where palmitoylation was interrogated only after the activation signal.

Recent studies have begun to explore the NLRP3 inflammasome in therapeutic contexts, particularly regarding treatments that target IL-1β for inflammatory diseases (Dinarello and van der Meer, 2013). However, the therapeutic potential of NLRP3 itself remains relatively underexplored. Its involvement in various autoimmune and metabolic disorders is well-established, yet effective strategies for regulating NLRP3 are still lacking. While inhibitors aimed at reducing IL-1β, such as Anakinra or Canakinumab, activity are commonly used in clinical practice, it is crucial to recognise that NLRP3 also generates IL-18, a cytokine that plays a significant role in maintaining homeostasis (Piccini *et al.*, 2008; Ramírez and Cañete, 2018). Upon release, IL-18 enhances the production of interferon-gamma (IFN-γ), promotes the differentiation of T helper 1 (Th1) cells, and contributes to inflammatory responses. Given the diverse functions of NLRP3, directly targeting it –rather than focusing solely on IL-1β – offers a more promising approach to alleviating the effects of chronic inflammatory conditions. Although MCC950 is currently the most promising NLRP3 inhibitor in development, our understanding of NLRP3 remains limited (Coll *et al.*, 2015). Interestingly, recent research has suggested that NLRP3 plays a critical role in tumour development, as chronic inflammation is a well-known contributor to tumorigenesis (Xu *et al.*, 2021). This creates a potential intersection with FASN, which is metabolically active in the tumour microenvironment and regulates NLRP3, offering a dual approach to targeting cancer development. It should be noted that while some studies indicate NLRP3's involvement in cancer progression, this relationship is hypothesised to be dualistic; in some contexts, NLRP3 may promote tumour development through inflammation, while in others it could exhibit tumour-suppressive effects by inducing pyroptosis in cancer cells.

6.2 The lipid profile of the canonical and non-canonical inflammasome

Previous research supports the notion that the lipid profile at the time of NLRP3 inflammasome activation significantly influences its dynamics. This context plays a critical role in shaping the activation processes of NLRP3. Here, untargeted mass spectrometry was used to examine two central mechanisms of NLRP3 activation and the lipid context during their activation in both canonical and non-canonical inflammasomes.

For the canonical inflammasome, the well-established LPS and ATP was used to induce priming and activation. Conversely, the non-canonical inflammasome was activated by

treating cells with the TLR4 ligand Pam3CSK3, followed by LPS transfection. These experiments were carefully controlled by adding additional samples with which the generated mass spec data was compared. Mass spectrometry has rapidly become a cornerstone of biological investigation due to its unmatched accuracy and precision in tracking the movement of lipids and other small molecules, as well as the expanding capabilities of proteomic mass spectrometry through peptide digests. The results generated in this research provide valuable insight into the activation mechanisms of both canonical and non-canonical inflammasomes, highlighting distinct lipid and molecular changes that contribute to their regulation and potential therapeutic targeting.

Despite its strengths, mass spectrometry traditionally faces limitations in quantification and identification. Untargeted mass spectrometry cannot provide quantification, meaning that the exact quantities of identified lipids, beyond their relative abundance, remain unknown. This limitation makes it challenging to determine which metabolic pathways are enriched or diminished in response to NLRP3 activation. However, this method enables comparisons between conditions and demonstrates changes across different scenarios, regardless of the actual measurable amounts. In this study, it was observed that the canonical inflammasome activation by ATP resulted in a significant shift in the total lipidome, primarily characterised by increased levels of ceramide and diacylglycerol (DAG). Ceramide has previously been implicated in inflammasome activation and has been reported to accumulate in response to NLRP3 activation. The findings in this work further validate this relationship. Ceramide is a bioactive sphingolipid suspected to play a role in cell death beyond its involvement in inflammation. Its role in the NLRP3 inflammasome is hypothesised to act as a secondary signalling messenger, inducing additional mitochondrial damage and ROS production. This connection has been explored through early ceramide production inhibition in rats using the inhibitor imipramine, which resulted in reduced markers of NLRP3 activation (Ouyang *et al.*, 2023).

In contrast, the role of DAG in inflammasome activation is less well understood. DAG has been implicated in NLRP3 activation through the phosphorylation of the Ser295 site(Hamilton *et al.*, 2022). Ser295 modification, mediated by DAG at the Golgi, clusters damaged mitochondria and promotes NLRP3 activation and release from MAMs. The spatial orientation of NLRP3 is critically important, and DAG may function to facilitate the localisation of NLRP3 to the Golgi (Zhang *et al.*, 2017a). This idea aligns with the findings presented in this thesis regarding the association of NLRP3 with the TGN during activation, suggesting that DAG could play a role in this association. While investigation of inflammasome activators other than ATP or transfected LPS was outside the scope of this study, it is plausible that various lipid classes

are remodelled depending on the activation signal received – nigericin or silica crystals could be a logical next step to confirm if ATP alone induced the changes reported in this thesis.

Greater detail emerged by comparing the lipid profiles of LPS and ATP combined versus ATP alone. Of particular note was NAE, which was observed to be less abundant known for its anti-inflammatory effects. NAE accumulates in tissues post-injury and typically acts to suppress inflammation; however, this effect may be overridden when macrophages are exposed to high ATP levels in the presence of LPS, fully activating the NLRP3 inflammasome and permitting pyroptosis. In canonical NLRP3-activated macrophages treated with FASN inhibitor C75, no single lipid class dominated, though several—including PC, PG, PE, and ceramide—are associated with membrane integrity. Furthermore, specific lipids uniquely found in activated NLRP3 cells, such as SM, CAR and plasmalogen PC-O (38:8), suggest a shift in lipid saturation, potentially linked to desaturation or oxidative stress. The lack of notable alterations in any single lipid class suggests that lipid remodelling or adjustments in the availability of existing lipids may compensate for FASN inhibition.

Testing specific lipid inhibitors of the identified pathways or employing mimetic LC-MS/MS to alter pathways could validate which lipid pathways are relevant to NLRP3 regulation. Limitations include the untargeted nature of the approach (which limits precise quantification), potential variation in inflammasome activation across cells and challenges in establishing causality within lipid networks. Nonetheless, this analysis provides a foundational insight into lipid regulators in NLRP3 activation, setting the stage for future studies on its role in inflammation-related metabolic disorders.

6.3 Concluding remarks

Recent breakthroughs in innate immunity have transformed what was once seen as a straightforward stimulus-response system into a complex, interwoven network of regulatory pathways, where cellular context influences the licensing of inflammatory responses. Among inflammasomes, NLRP3 is the most well-characterised, with established roles in autoimmune diseases, such as rheumatoid arthritis, and metabolic disorders like diabetes. Its ability to respond to diverse signals makes NLRP3 both an appealing drug target and a complex entity in terms of activation mechanisms. This work reveals that NLRP3 licensing depends on the essential enzyme FASN, further clarifying that FASN enables NLRP3 translocation to the Golgi, a step critical for its activation. This study also delineates the cellular context in which inflammasome activation occurs through untargeted lipidomics, which has revealed the lipid classes that are most influenced by the inflammasome activation and suggested new areas which could be explored to better understand how the inflammasome is regulated. By uncovering new aspects of NLRP3 function, this research lays the groundwork for identifying the precise steps necessary for inflammasome assembly and activation. Mapping the complex NLRP3 pathway will be a remarkable advancement, setting the tone for future research in innate immunity as it moves to the forefront of healthcare innovation.

Chapter 7

References

Chapter 7 References

Aachoui, Y. *et al.* (2013) 'Caspase-11 protects against bacteria that escape the vacuole', *Science (New York, N.Y.)*, 339(6122), pp. 975–978. doi: 10.1126/SCIENCE.1230751.

Abais, J. M. *et al.* (2015) 'Redox regulation of NLRP3 inflammasomes: ROS as trigger or effector?', *Antioxidants & redox signaling*, 22(13), pp. 1111–1129. doi: 10.1089/ARS.2014.5994.

Abudukelimu, A. *et al.* (2018) 'Predictable irreversible switching between acute and chronic inflammation', *Frontiers in Immunology*, 9(AUG), p. 1. doi: 10.3389/FIMMU.2018.01596/FULL.

Adhyaru, B. B. and Jacobson, T. A. (2018) 'Safety and efficacy of statin therapy', *Nature Reviews Cardiology 2018 15:12*, 15(12), pp. 757–769. doi: 10.1038/s41569-018-0098-5.

Adorni, M. P. *et al.* (2007) 'The roles of different pathways in the release of cholesterol from macrophages', *Journal of Lipid Research*. doi: 10.1194/jlr.M700274-JLR200.

Aganna, E. *et al.* (2002) 'Association of mutations in the NALP3/CIAS1/PYPAF1 gene with a broad phenotype including recurrent fever, cold sensitivity, sensorineural deafness, and AA amyloidosis', *Arthritis and rheumatism*, 46(9), pp. 2445–2452. doi: 10.1002/ART.10509.

Agostini, L. *et al.* (2004) 'NALP3 forms an IL-1beta-processing inflammasome with increased activity in Muckle-Wells autoinflammatory disorder', *Immunity*, 20(3), pp. 319–325. doi: 10.1016/S1074-7613(04)00046-9.

Ahern, Kevin. Indira, R. (2021) *15.10.3: Fatty Acid Synthesis - Chemistry LibreTexts*. Available at: https://chem.libretexts.org/Courses/Fullerton_College/Introductory_Biochemistry/15%3A_Lipids/15.10%3A_Lipid_Details/15.10.03%3A_Fatty_Acid_Synthesis (Accessed: 7 December 2024).

Akbal, A. *et al.* (2022) 'How location and cellular signaling combine to activate the NLRP3 inflammasome', *Cellular & Molecular Immunology 2022 19:11*, 19(11), pp. 1201–1214. doi: 10.1038/s41423-022-00922-w.

Aksentijevich, I. *et al.* (2002) 'De novo CIAS1 mutations, cytokine activation, and evidence for genetic heterogeneity in patients with neonatal-onset multisystem inflammatory disease (NOMID): a new member of the expanding family of pyrin-associated autoinflammatory diseases', *Arthritis and rheumatism*, 46(12), pp. 3340–3348. doi: 10.1002/ART.10688.

Alexopoulou, L. *et al.* (2001) 'Recognition of double-stranded RNA and activation of NF-κB by Toll-like receptor 3', 413(6857), pp. 732–738. doi: 10.1038/35099560.

Allen, D. R. and McWhinney, B. C. (2019) 'Quadrupole Time-of-Flight Mass Spectrometry: A Paradigm Shift in Toxicology Screening Applications', *The Clinical Biochemist Reviews*, 40(3), p. 135. doi: 10.33176/AACB-19-00023.

Amarante-Mendes, G. P. *et al.* (2018) 'Pattern recognition receptors and the host cell

death molecular machinery', *Frontiers in Immunology*, 9(OCT), p. 417707. doi: 10.3389/FIMMU.2018.02379/BIBTEX.

Amemiya-Kudo, M. *et al.* (2002) 'Transcriptional activities of nuclear SREBP-1a, -1c, and -2 to different target promoters of lipogenic and cholesterogenic genes', *Journal of Lipid Research*, 43(8), pp. 1220–1235. doi: 10.1194/JLR.M100417-JLR200.

Anand, P. K. (2020) 'Lipids, inflammasomes, metabolism, and disease', *Immunological Reviews*, 297(1), pp. 108–122. doi: 10.1111/imr.12891.

Anand, P. K., Malireddi, R. K. S. and Kanneganti, T. D. (2011) 'Role of the Nlrp3 Inflammasome in Microbial Infection', *Frontiers in Microbiology*, 2(FEB). doi: 10.3389/FMICB.2011.00012.

Andersen, C. J. (2022) 'Lipid Metabolism in Inflammation and Immune Function', *Nutrients*, 14(7). doi: 10.3390/NU14071414.

Andreeva, L. *et al.* (2021) 'NLRP3 cages revealed by full-length mouse NLRP3 structure control pathway activation', *Cell*, 184(26), pp. 6299-6312.e22. doi: 10.1016/J.CELL.2021.11.011.

Artetxe, I. *et al.* (2017) 'Does Ceramide Form Channels? The Ceramide-Induced Membrane Permeabilization Mechanism', *Biophysical journal*, 113(4), pp. 860–868. doi: 10.1016/J.BPJ.2017.06.071.

Assies, J. *et al.* (2014) 'Effects of oxidative stress on fatty acid- and one-carbon-metabolism in psychiatric and cardiovascular disease comorbidity', *Acta Psychiatrica Scandinavica*, 130(3), p. 163. doi: 10.1111/ACPS.12265.

Bai, S. *et al.* (2024) 'Pyroptosis leads to loss of centrosomal integrity in macrophages', *Cell Death Discovery 2024 10:1*, 10(1), pp. 1–14. doi: 10.1038/s41420-024-02093-1.

Baik, S. H. *et al.* (2023) 'Hexokinase dissociation from mitochondria promotes oligomerization of VDAC which facilitates NLRP3 inflammasome assembly and activation', *Science immunology*, 8(84), p. eade7652. doi: 10.1126/SCIIMMUNOL.ADE7652.

Balasubramanian, A. *et al.* (2024) 'The palmitoylation of gasdermin D directs its membrane translocation and pore formation during pyroptosis', 9(94). Available at: https://www.science.org/doi/10.1126/sciimmunol.adn1452 (Accessed: 21 September 2024).

Baroja-Mazo, A. *et al.* (2019) 'Early endosome autoantigen 1 regulates IL-1β release upon caspase-1 activation independently of gasdermin D membrane permeabilization', *Scientific reports*, 9(1). doi: 10.1038/S41598-019-42298-4.

Barritt, S. A., DuBois-Coyne, S. E. and Dibble, C. C. (2024) 'Coenzyme A biosynthesis: mechanisms of regulation, function and disease', *Nature Metabolism 2024 6:6*, 6(6), pp. 1008–1023. doi: 10.1038/s42255-024-01059-y.

Barry, R. *et al.* (2018) 'SUMO-mediated regulation of NLRP3 modulates inflammasome activity', *Nature Communications 2018 9:1*, 9(1), pp. 1–14. doi: 10.1038/s41467-018-05321-2.

Bauernfeind, F. *et al.* (2011) 'Cutting Edge: Reactive Oxygen Species Inhibitors

Block Priming, but Not Activation, of the NLRP3 Inflammasome', *The Journal of Immunology*. doi: 10.4049/jimmunol.1100613.

Bauernfeind, F. G. *et al.* (2009a) 'Cutting edge: NF-kappaB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression', *Journal of immunology (Baltimore, Md. : 1950)*, 183(2), pp. 787–791. doi: 10.4049/JIMMUNOL.0901363.

Bauernfeind, F. G. *et al.* (2009b) 'NF-kB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression', *Journal of immunology (Baltimore, Md. : 1950)*, 183(2), p. 787. doi: 10.4049/JIMMUNOL.0901363.

Bayer, B. -L and Föster, W. (1979) 'ACTIONS OF PROSTAGLANDIN PRECURSORS AND OTHER UNSATURATED FATTY ACIDS ON CONDUCTION TIME AND REFRACTORY PERIOD IN THE CAT HEART in situ', *British Journal of Pharmacology*, 66(2), pp. 191–195. doi: 10.1111/j.1476-5381.1979.tb13664.x.

Bianco, F. *et al.* (2009) 'Acid sphingomyelinase activity triggers microparticle release from glial cells', *The EMBO journal*, 28(8), pp. 1043–1054. doi: 10.1038/EMBOJ.2009.45.

Bittner, Z. A. *et al.* (2021) 'BTK operates a phospho-tyrosine switch to regulate NLRP3 inflammasome activity', *The Journal of experimental medicine*, 218(11). doi: 10.1084/JEM.20201656.

Blancaflor, E. B. *et al.* (2014) 'N-Acylethanolamines: lipid metabolites with functions in plant growth and development', *The Plant Journal*, 79(4), pp. 568–583. doi: 10.1111/TPJ.12427.

Bortolotti, P., Faure, E. and Kipnis, E. (2018) 'Inflammasomes in tissue damages and immune disorders after trauma', *Frontiers in Immunology*, 9(AUG), p. 339749. doi: 10.3389/FIMMU.2018.01900/BIBTEX.

Botos, I., Segal, D. M. and Davies, D. R. (2011) 'The structural biology of Toll-like receptors', *Structure (London, England : 1993)*, 19(4), pp. 447–459. doi: 10.1016/J.STR.2011.02.004.

Boucher, D. *et al.* (2018) 'Caspase-1 self-cleavage is an intrinsic mechanism to terminate inflammasome activity', *The Journal of Experimental Medicine*, 215(3), p. 827. doi: 10.1084/JEM.20172222.

Braga, T. T. *et al.* (2017) 'Soluble Uric Acid Activates the NLRP3 Inflammasome', *Scientific Reports 2017 7:1*, 7(1), pp. 1–14. doi: 10.1038/srep39884.

Brencicova, E. *et al.* (2013) 'Nucleic acids and endosomal pattern recognition: how to tell friend from foe?', *Frontiers in Cellular and Infection Microbiology*, 3(JUL), p. 37. doi: 10.3389/FCIMB.2013.00037.

Brinkmann, V. *et al.* (2004) 'Neutrophil extracellular traps kill bacteria', *Science (New York, N.Y.)*, 303(5663), pp. 1532–1535. doi: 10.1126/SCIENCE.1092385.

Brown, G. D., Willment, J. A. and Whitehead, L. (2018) 'C-type lectins in immunity and homeostasis', *Nature Reviews Immunology 2018 18:6*, 18(6), pp. 374–389. doi: 10.1038/s41577-018-0004-8.

Brown, M. S. and Goldstein, J. L. (1997) 'The SREBP Pathway: Regulation of Cholesterol Metabolism by Proteolysis of a Membrane-Bound Transcription Factor', *Cell*, 89(3), pp. 331–340. doi: 10.1016/S0092-8674(00)80213-5.

Broz, P., Von Moltke, J., *et al.* (2010) 'Differential requirement for Caspase-1 autoproteolysis in pathogen-induced cell death and cytokine processing', *Cell host & microbe*, 8(6), pp. 471–483. doi: 10.1016/J.CHOM.2010.11.007.

Broz, P., Newton, K., *et al.* (2010) 'Redundant roles for inflammasome receptors NLRP3 and NLRC4 in host defense against Salmonella', *The Journal of Experimental Medicine*, 207(8), p. 1745. doi: 10.1084/JEM.20100257.

Broz, P. and Dixit, V. M. (2016) 'Inflammasomes: mechanism of assembly, regulation and signalling', 16(7), pp. 407–420. Available at: https://www.nature.com/articles/nri.2016.58 (Accessed: 16 May 2024).

Bruce, J. S. and Salter, A. M. (1996) 'Metabolic fate of oleic acid, palmitic acid and stearic acid in cultured hamster hepatocytes.', *Biochemical Journal*, 316(Pt 3), p. 847. doi: 10.1042/BJ3160847.

Brunette, R. L. *et al.* (2012) 'Extensive evolutionary and functional diversity among mammalian AIM2-like receptors', *The Journal of experimental medicine*, 209(11), pp. 1969–1983. doi: 10.1084/JEM.20121960.

Brydges, S. D. *et al.* (2009) 'Inflammasome-mediated disease animal models reveal roles for innate but not adaptive immunity', *Immunity*, 30(6), pp. 875–887. doi: 10.1016/J.IMMUNI.2009.05.005.

Cahill, C. M. and Rogers, J. T. (2008) 'Interleukin (IL) 1β Induction of IL-6 Is Mediated by a Novel Phosphatidylinositol 3-Kinase-dependent AKT/IκB Kinase α Pathway Targeting Activator Protein-1', *The Journal of Biological Chemistry*, 283(38), p. 25900. doi: 10.1074/JBC.M707692200.

Cahoon, J., Yang, D. and Wang, P. (2022) 'The noncanonical inflammasome in health and disease', *Infectious Medicine*, 1(3), pp. 208–216. doi: 10.1016/J.IMJ.2022.09.001.

Cajka, T. and Fiehn, O. (2014) 'Comprehensive analysis of lipids in biological systems by liquid chromatography-mass spectrometry', *TrAC Trends in Analytical Chemistry*, 61, pp. 192–206. doi: 10.1016/J.TRAC.2014.04.017.

Calligaris, D. *et al.* (2014) 'Application of desorption electrospray ionization mass spectrometry imaging in breast cancer margin analysis', *Proceedings of the National Academy of Sciences of the United States of America*, 111(42), pp. 15184–15189. doi: 10.1073/PNAS.1408129111/-/DCSUPPLEMENTAL.

Carpenter, S. and O'Neill, L. A. J. (2024) 'From periphery to center stage: 50 years of advancements in innate immunity', *Cell*, 187(9), pp. 2030–2051. doi: 10.1016/J.CELL.2024.03.036.

Carroll, R. G. *et al.* (2018) 'An unexpected link between fatty acid synthase and cholesterol synthesis in proinflammatory macrophage activation', *Journal of Biological Chemistry*, 293(15), pp. 5509–5521. doi: 10.1074/jbc.RA118.001921.

Carta, G. *et al.* (2015) 'Dietary Triacylglycerols with Palmitic Acid in the sn-2 Position Modulate Levels of N-Acylethanolamides in Rat Tissues', *PLoS ONE*, 10(3). doi:

10.1371/JOURNAL.PONE.0120424.

Carta, G. *et al.* (2017a) 'Palmitic acid: Physiological role, metabolism and nutritional implications', *Frontiers in Physiology*, 8(NOV), p. 306122. doi: 10.3389/FPHYS.2017.00902/BIBTEX.

Carta, G. *et al.* (2017b) 'Palmitic Acid: Physiological Role, Metabolism and Nutritional Implications', *Frontiers in Physiology*, 8(NOV), p. 902. doi: 10.3389/FPHYS.2017.00902.

de Carvalho, R. V. H. *et al.* (2019) 'Leishmania Lipophosphoglycan Triggers Caspase-11 and the Non-canonical Activation of the NLRP3 Inflammasome', *Cell reports*, 26(2), p. 429. doi: 10.1016/J.CELREP.2018.12.047.

Case, C. L. (2011) 'Regulating Caspase-1 During Infection: Roles of NLRs, AIM2, and ASC', *The Yale Journal of Biology and Medicine*, 84(4), p. 333. Available at: https://pmc.ncbi.nlm.nih.gov/articles/PMC3238316/ (Accessed: 26 October 2024).

Case, C. L. *et al.* (2013) 'Caspase-11 stimulates rapid flagellin-independent pyroptosis in response to Legionella pneumophila', *Proceedings of the National Academy of Sciences of the United States of America*, 110(5), pp. 1851–1856. doi: 10.1073/PNAS.1211521110.

Caution, K. *et al.* (2015) 'Caspase-11 and caspase-1 differentially modulate actin polymerization via RhoA and Slingshot proteins to promote bacterial clearance', *Scientific Reports 2015 5:1*, 5(1), pp. 1–13. doi: 10.1038/srep18479.

Chamberlain, L. H. and Shipston, M. J. (2015) 'The Physiology of Protein S-acylation', *Physiological Reviews*, 95(2), p. 341. doi: 10.1152/PHYSREV.00032.2014.

Charles A Janeway, J. *et al.* (2001) 'Effector mechanisms in allergic reactions'. Available at: https://www.ncbi.nlm.nih.gov/books/NBK27112/ (Accessed: 2 December 2024).

Chen, J. and Chen, Z. J. (2018a) 'PtdIns4P on dispersed trans-Golgi network mediates NLRP3 inflammasome activation', *Nature 2018 564:7734*, 564(7734), pp. 71–76. doi: 10.1038/s41586-018-0761-3.

Chen, J. and Chen, Z. J. (2018b) 'PtdIns4P on Dispersed Trans-Golgi Network Mediates NLRP3 Inflammasome Activation', *Nature*, 564(7734), p. 71. doi: 10.1038/S41586-018-0761-3.

Chevriaux, A. *et al.* (2020) 'Cathepsin B Is Required for NLRP3 Inflammasome Activation in Macrophages, Through NLRP3 Interaction', *Frontiers in Cell and Developmental Biology*, 8. doi: 10.3389/FCELL.2020.00167/FULL.

Chi, C. *et al.* (2022) 'Concomitant Inhibition of FASN and SREBP Provides a Promising Therapy for CTCL', *Cancers*, 14(18). doi: 10.3390/CANCERS14184491/S1.

Choi, S. E. *et al.* (2011) 'Stimulation of Lipogenesis as Well as Fatty Acid Oxidation Protects against Palmitate-Induced INS-1 β-Cell Death', *Endocrinology*, 152(3), pp. 816–827. doi: 10.1210/EN.2010-0924.

Chomarat, P. *et al.* (2000) 'IL-6 switches the differentiation of monocytes from

dendritic cells to macrophages', *Nature Immunology 2000 1:6*, 1(6), pp. 510–514. doi: 10.1038/82763.

Chou, W. C. *et al.* (2021) 'Impact of intracellular innate immune receptors on immunometabolism', *Cellular & Molecular Immunology 2021 19:3*, 19(3), pp. 337–351. doi: 10.1038/s41423-021-00780-y.

Chu, L. H. *et al.* (2018) 'The oxidized phospholipid oxPAPC protects from septic shock by targeting the non-canonical inflammasome in macrophages', 9(1), pp. 1–16. Available at: https://www.nature.com/articles/s41467-018-03409-3 (Accessed: 28 December 2021).

Chuenchor, W. *et al.* (2014) 'Structures of pattern recognition receptors reveal molecular mechanisms of autoinhibition, ligand recognition and oligomerization', *Current opinion in immunology*, 26(1), pp. 14–20. doi: 10.1016/J.COI.2013.10.009.

Ciehanover, A., Hod, Y. and Hershko, A. (1978) 'A heat-stable polypeptide component of an ATP-dependent proteolytic system from reticulocytes', *Biochemical and Biophysical Research Communications*, 81(4), pp. 1100–1105. doi: 10.1016/0006-291X(78)91249-4.

Cockcroft, S. (2021) 'Mammalian lipids: structure, synthesis and function', *Essays in Biochemistry*, 65(5), p. 813. doi: 10.1042/EBC20200067.

Coll, R. C. *et al.* (2015) 'A small-molecule inhibitor of the NLRP3 inflammasome for the treatment of inflammatory diseases', *Nature medicine*, 21(3), pp. 248–257. doi: 10.1038/NM.3806.

Coll, R. C. *et al.* (2019) 'MCC950 directly targets the NLRP3 ATP-hydrolysis motif for inflammasome inhibition', *Nature Chemical Biology 2019 15:6*, 15(6), pp. 556–559. doi: 10.1038/s41589-019-0277-7.

Coll, R. C., Holley, C. L. and Schroder, K. (2018) 'Mitochondrial DNA synthesis fuels NLRP3 inflammasome', *Cell Research*, 28(11), p. 1046. doi: 10.1038/S41422-018-0093-8.

Collins, J. M. *et al.* (2010) 'De novo lipogenesis and stearoyl-CoA desaturase are coordinately regulated in the human adipocyte and protect against palmitate-induced cell injury', *The Journal of biological chemistry*, 285(9), pp. 6044–6052. doi: 10.1074/JBC.M109.053280.

Colomar, A. *et al.* (2003) 'Maturation and release of interleukin-1beta by lipopolysaccharide-primed mouse Schwann cells require the stimulation of P2X7 receptors', *The Journal of biological chemistry*, 278(33), pp. 30732–30740. doi: 10.1074/JBC.M304534200.

Corrêa, R. *et al.* (2020) 'Lysophosphatidylcholine Induces NLRP3 Inflammasome-Mediated Foam Cell Formation and Pyroptosis in Human Monocytes and Endothelial Cells', *Frontiers in Immunology*, 10. Available at: www.frontiersin.org (Accessed: 15 September 2024).

Cortez-Retamozo, V. *et al.* (2012) 'Origins of tumor-associated macrophages and neutrophils', *Proceedings of the National Academy of Sciences of the United States of America*, 109(7), pp. 2491–2496. doi: 10.1073/PNAS.1113744109/SUPPL_FILE/PNAS.201113744SI.PDF.

Coskun, O. (2016) ‘Separation techniques: Chromatography’, *Northern Clinics of Istanbul*, 3(2), p. 156. doi: 10.14744/NCI.2016.32757.

Cullis, P. R. and De Kruijff, B. (1979) ‘Lipid polymorphism and the functional roles of lipids in biological membranes’, *Biochimica et biophysica acta*, 559(4), pp. 399–420. doi: 10.1016/0304-4157(79)90012-1.

Cuyàs, E. *et al.* (2024) ‘Fatty acid synthase (FASN) is a tumor-cell-intrinsic metabolic checkpoint restricting T-cell immunity’, *Cell Death Discovery 2024 10:1*, 10(1), pp. 1–17. doi: 10.1038/s41420-024-02184-z.

Dang, E. V. *et al.* (2017) ‘Oxysterol restraint of cholesterol synthesis prevents AIM2 inflammasome activation’, *Cell*, 171(5), p. 1057. doi: 10.1016/J.CELL.2017.09.029.

Daniels, M. J. D. *et al.* (2016) ‘Fenamate NSAIDs inhibit the NLRP3 inflammasome and protect against Alzheimer’s disease in rodent models’, *Nature Communications 2016 7:1*, 7(1), pp. 1–10. doi: 10.1038/ncomms12504.

Davda, D. *et al.* (2013) ‘Profiling targets of the irreversible palmitoylation inhibitor 2-bromopalmitate’, *ACS Chemical Biology*, 8(9), pp. 1912–1917. doi: 10.1021/CB400380S/SUPPL_FILE/CB400380S_SI_002.PDF.

DeBose-Boyd, R. A. *et al.* (1999) ‘Transport-dependent proteolysis of SREBP: Relocation of Site-1 protease from Golgi to ER obviates the need for SREBP transport to Golgi’, *Cell*, 99(7), pp. 703–712. doi: 10.1016/S0092-8674(00)81668-2.

DeBose-Boyd, R. A. and Ye, J. (2018) ‘SREBPs in Lipid Metabolism, Insulin Signaling, and Beyond’, *Trends in Biochemical Sciences*. NIH Public Access, pp. 358–368. doi: 10.1016/j.tibs.2018.01.005.

Degen, M. *et al.* (2023) ‘Structural basis of NINJ1-mediated plasma membrane rupture in cell death’, *Nature*, 618(7967), pp. 1065–1071. doi: 10.1038/s41586-023-05991-z.

Dekker, C. *et al.* (2021) ‘Crystal Structure of NLRP3 NACHT Domain With an Inhibitor Defines Mechanism of Inflammasome Inhibition’, *Journal of Molecular Biology*, 433(24), p. 167309. doi: 10.1016/J.JMB.2021.167309.

Dempsey, L. A. (2020) ‘Sirtuin regulation of NLRP3’, *Nature Immunology 2020 21:4*, 21(4), pp. 358–358. doi: 10.1038/s41590-020-0657-x.

Desaki, Y. *et al.* (2006) ‘Bacterial lipopolysaccharides induce defense responses associated with programmed cell death in rice cells’, *Plant & cell physiology*, 47(11), pp. 1530–1540. doi: 10.1093/PCP/PCL019.

Dewulf, J. P. *et al.* (2019) ‘The synthesis of branched-chain fatty acids is limited by enzymatic decarboxylation of ethyl- and methylmalonyl-CoA’, *Biochemical Journal*, 476(16), p. 2427. doi: 10.1042/BCJ20190500.

DeYoung, K. L. *et al.* (1997) ‘Cloning a novel member of the human interferon-inducible gene family associated with control of tumorigenicity in a model of human melanoma’, *Oncogene*. doi: 10.1038/sj.onc.1201206.

Dinarello, C. A. *et al.* (2013) ‘Interleukin-18 and IL-18 binding protein’, *Frontiers in Immunology*, 4(OCT), p. 57626. doi: 10.3389/FIMMU.2013.00289/BIBTEX.

Dinarello, C. A. (2018) ‘Overview of the IL-1 family in innate inflammation and acquired immunity’, *Immunological reviews*, 281(1), p. 8. doi: 10.1111/IMR.12621.

Dinarello, C. A. and van der Meer, J. W. M. (2013) ‘Treating inflammation by blocking interleukin-1 in humans’, *Seminars in immunology*, 25(6), pp. 469–484. doi: 10.1016/J.SMIM.2013.10.008.

Domingo-Fernández, R. *et al.* (2017) ‘The intracellular chloride channel proteins CLIC1 and CLIC4 induce IL-1β transcription and activate the NLRP3 inflammasome’, *The Journal of biological chemistry*, 292(29), pp. 12077–12087. doi: 10.1074/JBC.M117.797126.

Dong, Z. *et al.* (2020) ‘Palmitic acid stimulates NLRP3 inflammasome activation through TLR4-NF-κB signal pathway in hepatic stellate cells’, *Annals of Translational Medicine*, 8(5), pp. 168–168. doi: 10.21037/ATM.2020.02.21.

Downes, C. P. and Currie, R. A. (1998) ‘Lipid signalling.’, *Current biology : CB*, 8(24), pp. R865–R867. doi: 10.1016/s0960-9822(07)00546-5.

Downs, K. P. *et al.* (2020) ‘An overview of the non-canonical inflammasome’, *Molecular Aspects of Medicine*, 76, p. 100924. Available at: http://creativecommons.org/licenses/by/4.0/ (Accessed: 22 May 2024).

Draper, J. M., Xia, Z. and Smith, C. D. (2007) ‘Cellular palmitoylation and trafficking of lipidated peptides’, *Journal of Lipid Research*, 48(8), pp. 1873–1884. doi: 10.1194/jlr.M700179-JLR200.

Du, G. *et al.* (2023) ‘ROS-dependent palmitoylation is an obligate licensing modification for GSDMD pore formation’, *bioRxiv*, p. 2023.03.07.531538. doi: 10.1101/2023.03.07.531538.

Du, G. *et al.* (2024) ‘ROS-dependent S-palmitoylation activates cleaved and intact gasdermin D’, *Nature 2024 630:8016*, 630(8016), pp. 437–446. doi: 10.1038/s41586-024-07373-5.

Dueñas, M. E. *et al.* (2023) ‘Advances in high-throughput mass spectrometry in drug discovery’, *EMBO Molecular Medicine*, 15(1). doi: 10.15252/EMMM.202114850.

Duewell, P. *et al.* (2010a) ‘NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals’, *Nature*, 464(7293), pp. 1357–1361. doi: 10.1038/NATURE08938.

Duewell, P. *et al.* (2010b) ‘NLRP3 inflammasomes are required for atherogenesis and activated by cholesterol crystals’, *Nature*. doi: 10.1038/nature08938.

Duncan, J. A. *et al.* (2007) ‘Cryopyrin/NALP3 binds ATP/dATP, is an ATPase, and requires ATP binding to mediate inflammatory signaling’, *Proceedings of the National Academy of Sciences of the United States of America*, 104(19), pp. 8041–8046. doi: 10.1073/PNAS.0611496104.

Duncan, J. A. and Canna, S. W. (2018) ‘The NLRC4 Inflammasome ORIGINS NLRC4 was originally described by Poyet and colleagues as a pro-apoptotic protein. Searching for structural homologues of APAF1: a protein known to activate apoptotic caspases in response to cytosolic cytochrome c, and containing a Caspase-Activation and Recruitment Domain (CARD) and ATP-binding site 2 , they identified a protein capable of HHS Public Access’, *Immunol Rev*, 281(1), pp. 115–123. doi:

10.1111/imr.12607.

Duran-Salgado, M. B. (2014) ‘Diabetic nephropathy and inflammation’, *World Journal of Diabetes*, 5(3), p. 393. doi: 10.4239/wjd.v5.i3.393.

E.G. Krebs, Kent, A. B. E. H. F. (1958) ‘The muscle phosphorylase b kinase reaction.’, *The Journal of biological chemistry*, 231(1), pp. 73–83. doi: 10.1016/s0021-9258(19)77286-x.

Eichelberger, K. R. and Goldman, W. E. (2020) ‘Manipulating neutrophil degranulation as a bacterial virulence strategy’, *PLoS Pathogens*, 16(12), p. e1009054. doi: 10.1371/JOURNAL.PPAT.1009054.

Elliott, E. I. *et al.* (2018) ‘Cutting Edge: Mitochondrial Assembly of the NLRP3 Inflammasome Complex Is Initiated at Priming’, *Journal of immunology (Baltimore, Md. : 1950)*, 200(9), pp. 3047–3052. doi: 10.4049/JIMMUNOL.1701723.

Elliott, E. I. and Sutterwala, F. S. (2015) ‘Initiation and perpetuation of NLRP3 inflammasome activation and assembly’, *Immunological Reviews*, 265(1), pp. 35–52. doi: 10.1111/IMR.12286.

Elliott, J. M. *et al.* (2009) ‘Crystal Structure of Procaspase-1 Zymogen Domain Reveals Insight into Inflammatory Caspase Autoactivation’, *The Journal of Biological Chemistry*, 284(10), p. 6546. doi: 10.1074/JBC.M806121200.

Epand, R. M., Fuller, N. and Rand, R. P. (1996) ‘Role of the position of unsaturation on the phase behavior and intrinsic curvature of phosphatidylethanolamines’, *Biophysical journal*, 71(4), pp. 1806–1810. doi: 10.1016/S0006-3495(96)79381-5.

Ernst, A. M. *et al.* (2018) ‘S-Palmitoylation Sorts Membrane Cargo for Anterograde Transport in the Golgi’, *Developmental cell*, 47(4), pp. 479-493.e7. doi: 10.1016/J.DEVCEL.2018.10.024.

Feerick, C. L. and McKernan, D. P. (2017) ‘Understanding the regulation of pattern recognition receptors in inflammatory diseases – a “Nod” in the right direction’, *Immunology*, pp. 237–247. doi: 10.1111/imm.12677.

Feingold, K. R. and Grunfeld, C. (2000) *Introduction to Lipids and Lipoproteins*, *Endotext*.

Fernandes-Alnemri, T. *et al.* (2009a) ‘AIM2 activates the inflammasome and cell death in response to cytoplasmic DNA’, *Nature*. doi: 10.1038/nature07710.

Fernandes-Alnemri, T. *et al.* (2009b) ‘AIM2 activates the inflammasome and cell death in response to cytoplasmic DNA’, *Nature*, 458(7237), p. 509. doi: 10.1038/NATURE07710.

Fhu, C. W. and Ali, A. (2020) ‘Fatty Acid Synthase: An Emerging Target in Cancer’, *Molecules*, 25(17). doi: 10.3390/MOLECULES25173935.

Fischer, E, H. Krebs, E. G. (1955) ‘Conversion of phosphorylase b to phosphorylase a in muscle extracts.’, *The Journal of biological chemistry*, 216(1), pp. 121–132. doi: 10.1016/s0021-9258(19)52289-x.

Fitrolaki, M. D. *et al.* (2016) ‘Increased extracellular heat shock protein 90α in severe sepsis and SIRS associated with multiple organ failure and related to acute

inflammatory-metabolic stress response in children', *Medicine*, 95(35). doi: 10.1097/MD.0000000000004651.

Flavin, R. *et al.* (2010) 'Fatty acid synthase as a potential therapeutic target in cancer', *Future oncology (London, England)*, 6(4), p. 551. doi: 10.2217/FON.10.11.

Florance, I. and Ramasubbu, S. (2023) 'Current Understanding on the Role of Lipids in Macrophages and Associated Diseases', *International Journal of Molecular Sciences*, 24(1). doi: 10.3390/IJMS24010589.

Fu, J., Schroder, K. and Wu, H. (2024) 'Mechanistic insights from inflammasome structures', *Nature Reviews Immunology 2024 24:7*, 24(7), pp. 518–535. doi: 10.1038/s41577-024-00995-w.

Fujii, S. I. *et al.* (2004) 'The Linkage of Innate to Adaptive Immunity via Maturing Dendritic Cells In Vivo Requires CD40 Ligation in Addition to Antigen Presentation and CD80/86 Costimulation', *The Journal of Experimental Medicine*, 199(12), p. 1607. doi: 10.1084/JEM.20040317.

Fukata, Y. *et al.* (2013) 'Local palmitoylation cycles define activity-regulated postsynaptic subdomains', *Journal of Cell Biology*, 202(1), pp. 145–161. doi: 10.1083/JCB.201302071/VIDEO-5.

Fukata, Y., Bredt, D. S. and Fukata, M. (2006) 'Protein Palmitoylation by DHHC Protein Family', *The Dynamic Synapse: Molecular Methods in Ionotropic Receptor Biology*, pp. 83–90. doi: 10.1201/9780203486283.ch5.

Galli, G. *et al.* (2022) 'Fas/CD95 Signaling Pathway in Damage-Associated Molecular Pattern (DAMP)-Sensing Receptors', *Cells*, 11(9), p. 1438. doi: 10.3390/CELLS11091438.

Galli, S. J., Borregaard, N. and Wynn, T. A. (2011) 'Phenotypic and functional plasticity of cells of innate immunity: macrophages, mast cells and neutrophils', *Nature Immunology 2011 12:11*, 12(11), pp. 1035–1044. doi: 10.1038/ni.2109.

Gallo, R. L. and Hooper, L. V. (2012) 'Epithelial antimicrobial defence of the skin and intestine', *Nature reviews. Immunology*, 12(7), p. 503. doi: 10.1038/NRI3228.

Galván-Peña, S. *et al.* (2019) 'Malonylation of GAPDH is an inflammatory signal in macrophages', *Nature Communications 2019 10:1*, 10(1), pp. 1–11. doi: 10.1038/s41467-018-08187-6.

García-Sastre, A. (2002) 'Mechanisms of inhibition of the host interferon α/β-mediated antiviral responses by viruses', *Microbes and Infection*, 4(6), pp. 647–655. doi: 10.1016/S1286-4579(02)01583-6.

Gasteiger, G. *et al.* (2017) 'Cellular Innate Immunity: An Old Game with New Players', *Journal of Innate Immunity*, 9(2), pp. 111–125. doi: 10.1159/000453397.

Géhin, C., Fowler, S. J. and Trivedi, D. K. (2023) 'Chewing the fat: How lipidomics is changing our understanding of human health and disease in 2022', *Analytical science advances*, 4(3–4), pp. 104–131. doi: 10.1002/ANSA.202300009.

Ghait, M. *et al.* (2023) 'The non-canonical inflammasome activators Caspase-4 and Caspase-5 are differentially regulated during immunosuppression-associated organ damage', *Frontiers in Immunology*, 14, p. 1239474. doi:

10.3389/FIMMU.2023.1239474/BIBTEX.

Gierlikowska, B., Gierlikowski, W. and Demkow, U. (2020) ‘Alantolactone Enhances the Phagocytic Properties of Human Macrophages and Modulates Their Proinflammatory Functions’, *Frontiers in pharmacology*, 11. doi: 10.3389/FPHAR.2020.01339.

Ginefra, P., Lorusso, G. and Vannini, N. (2020) ‘Innate Immune Cells and Their Contribution to T-Cell-Based Immunotherapy’, *International Journal of Molecular Sciences*, 21(12), p. 4441. doi: 10.3390/IJMS21124441.

Ginhoux, F. *et al.* (2015) ‘New insights into the multidimensional concept of macrophage ontogeny, activation and function’, *Nature Immunology 2015 17:1*, 17(1), pp. 34–40. doi: 10.1038/ni.3324.

Ginhoux, F. and Jung, S. (2014) ‘Monocytes and macrophages: developmental pathways and tissue homeostasis’, *Nature Reviews Immunology 2014 14:6*, 14(6), pp. 392–404. doi: 10.1038/nri3671.

Glish, G. L. and Vachet, R. W. (2003) ‘The basics of mass spectrometry in the twenty-first century’, *Nature Reviews Drug Discovery 2003 2:2*, 2(2), pp. 140–150. doi: 10.1038/nrd1011.

Godi, A. *et al.* (1999) ‘ARF mediates recruitment of PtdIns-4-OH kinase-beta and stimulates synthesis of PtdIns(4,5)P2 on the Golgi complex’, *Nature cell biology*, 1(5), pp. 280–287. doi: 10.1038/12993.

Gong, Z. *et al.* (2015) ‘Curcumin suppresses NLRP3 inflammasome activation and protects against LPS-induced septic shock’, *Molecular nutrition & food research*, 59(11), pp. 2132–2142. doi: 10.1002/MNFR.201500316.

Gordy, C. *et al.* (2011) ‘Regulation of steady-state neutrophil homeostasis by macrophages’, *Blood*, 117(2), pp. 618–629. doi: 10.1182/BLOOD-2010-01-265959.

Goudot, C. *et al.* (2017) ‘Aryl Hydrocarbon Receptor Controls Monocyte Differentiation into Dendritic Cells versus Macrophages’, *Immunity*, 47(3), pp. 582-596.e6. doi: 10.1016/J.IMMUNI.2017.08.016/ATTACHMENT/BB466C13-A21D-4D02-B4E3-BD1A1810E49F/MMC5.PDF.

Grassmé, H. *et al.* (2001) ‘CD95 signaling via ceramide-rich membrane rafts’, *The Journal of biological chemistry*, 276(23), pp. 20589–20596. doi: 10.1074/JBC.M101207200.

Grassmé, H. *et al.* (2014) ‘Regulation of the inflammasome by ceramide in cystic fibrosis lungs’, *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology*, 34(1), pp. 45–55. doi: 10.1159/000362983.

Gray, E. E. *et al.* (2016) ‘The AIM2-like receptors are dispensable for the interferon response to intracellular DNA’, *Immunity*, 45(2), p. 255. doi: 10.1016/J.IMMUNI.2016.06.015.

Green, J. P. *et al.* (2018) ‘Chloride regulates dynamic NLRP3-dependent ASC oligomerization and inflammasome priming’, *Proceedings of the National Academy of Sciences of the United States of America*, 115(40), pp. E9371–E9380. doi: 10.1073/PNAS.1812744115/SUPPL_FILE/PNAS.1812744115.SAPP.PDF.

Green, J. P. *et al.* (2020) 'LRRC8A is essential for hypotonicity-, but not for damp-induced nlrp3 inflammasome activation', *eLife*, 9, pp. 1–22. doi: 10.7554/ELIFE.59704.

Gritsenko, A. *et al.* (2020) 'Priming Is Dispensable for NLRP3 Inflammasome Activation in Human Monocytes In Vitro', *Frontiers in immunology*, 11. doi: 10.3389/FIMMU.2020.565924.

Guan, X. and Fierke, C. A. (2011) 'Understanding Protein Palmitoylation: Biological Significance and Enzymology', *Science China. Chemistry*, 54(12), p. 1888. doi: 10.1007/S11426-011-4428-2.

Guerbette, T. *et al.* (2024) 'Saturated fatty acids differently affect mitochondrial function and the intestinal epithelial barrier depending on their chain length in the in vitro model of IPEC-J2 enterocytes', *Frontiers in Cell and Developmental Biology*, 12, p. 1266842. doi: 10.3389/FCELL.2024.1266842/BIBTEX.

Guo, C. *et al.* (2018) 'Cholesterol Homeostatic Regulator SCAP-SREBP2 Integrates NLRP3 Inflammasome Activation and Cholesterol Biosynthetic Signaling in Macrophages', *Immunity*, 49(5), pp. 842-856.e7. Available at: https://pubmed.ncbi.nlm.nih.gov/30366764/ (Accessed: 4 December 2021).

Gurung, P. *et al.* (2012) 'Toll or interleukin-1 receptor (TIR) domain-containing adaptor inducing interferon-β (TRIF)-mediated caspase-11 protease production integrates Toll-like receptor 4 (TLR4) protein- and Nlrp3 inflammasome-mediated host defense against enteropathogens', *The Journal of biological chemistry*, 287(41), pp. 34474–34483. doi: 10.1074/JBC.M112.401406.

Gurung, P. *et al.* (2014) 'FADD and Caspase-8 Mediate Priming and Activation of the Canonical and Noncanonical Nlrp3 Inflammasomes', *The Journal of Immunology*. doi: 10.4049/jimmunol.1302839.

Gurung, P. *et al.* (2015) 'Chronic TLR Stimulation Controls NLRP3 Inflammasome Activation through IL-10 Mediated Regulation of NLRP3 Expression and Caspase-8 Activation', *Scientific Reports*, 5. doi: 10.1038/SREP14488.

Gust, A. A. *et al.* (2007) 'Bacteria-derived Peptidoglycans Constitute Pathogen-associated Molecular Patterns Triggering Innate Immunity in Arabidopsis', *Journal of Biological Chemistry*, 282(44), pp. 32338–32348. doi: 10.1074/JBC.M704886200.

Haldar, M. and Murphy, K. M. (2014) 'Origin, Development, and Homeostasis of Tissue-resident Macrophages', *Immunological reviews*, 262(1), p. 25. doi: 10.1111/IMR.12215.

Hall, C. J. *et al.* (2018) 'Blocking fatty acid–fueled mROS production within macrophages alleviates acute gouty inflammation', *The Journal of Clinical Investigation*, 128(5), pp. 1752–1771. doi: 10.1172/JCI94584.

Hamilton, C. *et al.* (2022) 'NLRP3 Inflammasome Priming and Activation Are Regulated by a Phosphatidylinositol-Dependent Mechanism', *ImmunoHorizons*, 6(8). doi: 10.4049/IMMUNOHORIZONS.2200058.

Hamilton, C. and Anand, P. K. (2019) 'Right place, right time: localisation and assembly of the NLRP3 inflammasome', *F1000Research 2019 8:676*, 8, p. 676. doi: 10.12688/f1000research.18557.1.

Han, J. and Kaufman, R. J. (2016) ‘Thematic Review Series: Lipotoxicity: Many Roads to Cell Dysfunction and Cell Death: The role of ER stress in lipid metabolism and lipotoxicity’, *Journal of Lipid Research*, 57(8), p. 1329. doi: 10.1194/JLR.R067595.

Han, M. S. *et al.* (2013) ‘JNK expression by macrophages promotes obesity-induced insulin resistance and inflammation’, *Science (New York, N.Y.)*, 339(6116), pp. 218–222. doi: 10.1126/SCIENCE.1227568.

Hancock, J. F., Paterson, H. and Marshall, C. J. (1990) ‘A polybasic domain or palmitoylation is required in addition to the CAAX motif to localize p21ras to the plasma membrane’, *Cell*, 63(1), pp. 133–139. doi: 10.1016/0092-8674(90)90294-O.

Hannah, V. C. *et al.* (2001) ‘Unsaturated Fatty Acids Down-regulate SREBP Isoforms 1a and 1c by Two Mechanisms in HEK-293 Cells’, *Journal of Biological Chemistry*, 276(6), pp. 4365–4372. doi: 10.1074/JBC.M007273200.

Hannun, Y. A. and Luberto, C. (2000) ‘Ceramide in the eukaryotic stress response’, *Trends in Cell Biology*, 10(2), pp. 73–80. doi: 10.1016/S0962-8924(99)01694-3/ASSET/71244428-1B8B-4B64-B3AE-962F0F2C3949/MAIN.ASSETS/GR3.SML.

Harber, K. J. *et al.* (2020) ‘Succinate Is an Inflammation-Induced Immunoregulatory Metabolite in Macrophages’, *Metabolites*, 10(9), pp. 1–14. doi: 10.3390/METABO10090372.

Hardwicke, M. A. *et al.* (2014) ‘A human fatty acid synthase inhibitor binds β-ketoacyl reductase in the keto-substrate site’, *Nature Chemical Biology 2014 10:9*, 10(9), pp. 774–779. doi: 10.1038/nchembio.1603.

Hata, A. N. and Breyer, R. M. (2004) ‘Pharmacology and signaling of prostaglandin receptors: multiple roles in inflammation and immune modulation’, *Pharmacology & therapeutics*, 103(2), pp. 147–166. doi: 10.1016/J.PHARMTHERA.2004.06.003.

Hawiger, D. *et al.* (2001) ‘Dendritic Cells Induce Peripheral T Cell Unresponsiveness under Steady State Conditions in Vivo’, *The Journal of Experimental Medicine*, 194(6), p. 769. doi: 10.1084/JEM.194.6.769.

Hay, R. T. (2013) ‘Decoding the SUMO signal’, *Biochemical Society Transactions*, 41(2), pp. 463–473. doi: 10.1042/BST20130015.

Hayashi, F. *et al.* (2001) ‘The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5’, *Nature*, 410(6832), pp. 1099–1103. doi: 10.1038/35074106.

He, M. *et al.* (2020) ‘An Acetylation Switch of the NLRP3 Inflammasome Regulates Aging-Associated Chronic Inflammation and Insulin Resistance’, *Cell metabolism*, 31(3), pp. 580-591.e5. doi: 10.1016/J.CMET.2020.01.009.

He, W. T. *et al.* (2015) ‘Gasdermin D is an executor of pyroptosis and required for interleukin-1β secretion’, *Cell Research 2015 25:12*, 25(12), pp. 1285–1298. doi: 10.1038/cr.2015.139.

He, Y. *et al.* (2016) ‘NEK7 is an essential mediator of NLRP3 activation downstream of potassium efflux’, 530(7590), pp. 354–357. doi: 10.1038/nature16959.

He, Y., Hara, H. and Núñez, G. (2016) ‘Mechanism and Regulation of NLRP3

Inflammasome Activation', *Trends in Biochemical Sciences*, 41(12), pp. 1012–1021. doi: 10.1016/J.TIBS.2016.09.002.

Heil, F. *et al.* (2004) 'Species-Specific Recognition of Single-Stranded RNA via Toll-like Receptor 7 and 8', *Science*. doi: 10.1126/science.1093620.

Helms, J. B. and Zurzolo, C. (2004) 'Lipids as Targeting Signals: Lipid Rafts and Intracellular Trafficking', *Traffic*, 5(4), pp. 247–254. doi: 10.1111/J.1600-0854.2004.0181.X.

Heneka, M. T. *et al.* (2012) 'NLRP3 is activated in Alzheimer's disease and contributes to pathology in APP/PS1 mice', *Nature 2012 493:7434*, 493(7434), pp. 674–678. doi: 10.1038/nature11729.

Hernandez-Cuellar, E. *et al.* (2012) 'Cutting edge: nitric oxide inhibits the NLRP3 inflammasome', *Journal of immunology (Baltimore, Md. : 1950)*, 189(11), pp. 5113–5117. doi: 10.4049/JIMMUNOL.1202479.

Hershko, A. *et al.* (1983) 'Components of ubiquitin-protein ligase system. Resolution, affinity purification, and role in protein breakdown.', *Journal of Biological Chemistry*, 258(13), pp. 8206–8214. doi: 10.1016/s0021-9258(20)82050-x.

Hiemstra, P. S., McCray, P. B. and Bals, R. (2015) 'The innate immune function of airway epithelial cells in inflammatory lung disease', *The European respiratory journal*, 45(4), p. 1150. doi: 10.1183/09031936.00141514.

Hivroz, C. *et al.* (2012) 'Crosstalk between T lymphocytes and dendritic cells', *Critical reviews in immunology*, 32(2), pp. 139–155. doi: 10.1615/CRITREVIMMUNOL.V32.I2.30.

Hochheiser, I. V. *et al.* (2022) 'Structure of the NLRP3 decamer bound to the cytokine release inhibitor CRID3', *Nature 2022 604:7904*, 604(7904), pp. 184–189. doi: 10.1038/s41586-022-04467-w.

Hoffman, H. M. *et al.* (2001) 'Mutation of a new gene encoding a putative pyrin-like protein causes familial cold autoinflammatory syndrome and Muckle–Wells syndrome', *Nature genetics*, 29(3), p. 301. doi: 10.1038/NG756.

Hohensinner, P. J. *et al.* (2021) 'Pharmacological inhibition of fatty acid oxidation reduces atherosclerosis progression by suppression of macrophage NLRP3 inflammasome activation', *Biochemical Pharmacology*, 190, p. 114634. doi: 10.1016/J.BCP.2021.114634.

Holzlechner, M., Eugenin, E. and Prideaux, B. (2019) 'Mass spectrometry imaging to detect lipid biomarkers and disease signatures in cancer', *Cancer Reports*, 2(6). doi: 10.1002/CNR2.1229.

Hooftman, A. *et al.* (2020) 'The Immunomodulatory Metabolite Itaconate Modifies NLRP3 and Inhibits Inflammasome Activation', *Cell metabolism*, 32(3), pp. 468-478.e7. doi: 10.1016/J.CMET.2020.07.016.

Hornung, V. *et al.* (2008) 'Silica crystals and aluminum salts activate the NALP3 inflammasome through phagosomal destabilization', 9(8), pp. 847–856. Available at: https://pubmed.ncbi.nlm.nih.gov/18604214/ (Accessed: 23 September 2024).

Hornung, V. *et al.* (2009) 'AIM2 recognizes cytosolic dsDNA and forms a caspase-1-

activating inflammasome with ASC', *Nature 2009 458:7237*, 458(7237), pp. 514–518. doi: 10.1038/nature07725.

Hotamisligil, G. S. (2006) 'Inflammation and metabolic disorders', *Nature 2006 444:7121*, 444(7121), pp. 860–867. doi: 10.1038/nature05485.

Hou, X. X. *et al.* (2018) 'Purinergic 2X7 Receptor is Involved in the Podocyte Damage of Obesity-Related Glomerulopathy via Activating Nucleotide-Binding and Oligomerization Domain-Like Receptor Protein 3 Inflammasome', *Chinese medical journal*, 131(22), pp. 2713–2725. doi: 10.4103/0366-6999.245270.

Hu, Z. *et al.* (2013) 'Crystal structure of NLRC4 reveals its autoinhibition mechanism', *Science (New York, N.Y.)*, 341(6142), pp. 172–175. doi: 10.1126/SCIENCE.1236381.

Huang, Y. *et al.* (2022) 'Inflammasome Activation and Pyroptosis via a Lipid-regulated SIRT1-p53-ASC Axis in Macrophages From Male Mice and Humans', *Endocrinology*, 163(4). doi: 10.1210/ENDOCR/BQAC014.

Humphries, F. *et al.* (2018) 'The E3 ubiquitin ligase Pellino2 mediates priming of the NLRP3 inflammasome', *Nature Communications 2018 9:1*, 9(1), pp. 1–17. doi: 10.1038/s41467-018-03669-z.

Ikonen, E. (2008) 'Cellular cholesterol trafficking and compartmentalization', *Nature Reviews Molecular Cell Biology 2008 9:2*, 9(2), pp. 125–138. doi: 10.1038/nrm2336.

Istvan, E. S. and Deisenhofer, J. (2001) 'Structural mechanism for statin inhibition of HMG-CoA reductase', *Science*. doi: 10.1126/science.1059344.

Ito, M. *et al.* (2015) 'Bruton's tyrosine kinase is essential for NLRP3 inflammasome activation and contributes to ischaemic brain injury', *Nature communications*, 6. doi: 10.1038/NCOMMS8360.

Iyer, S S. *et al.* (2013) 'Mitochondrial cardiolipin is required for Nlrp3 inflammasome activation', *Immunity*, 39(2), pp. 311–323. Available at: https://pubmed.ncbi.nlm.nih.gov/23954133/ (Accessed: 23 May 2024).

Iyer, Shankar S. *et al.* (2013a) 'Mitochondrial cardiolipin is required for Nlrp3 inflammasome activation', *Immunity*, 39(2), pp. 311–323. doi: 10.1016/J.IMMUNI.2013.08.001/ATTACHMENT/49FA7227-5E6D-4958-8378-E9A4186CAB9F/MMC1.PDF.

Iyer, Shankar S. *et al.* (2013b) 'Mitochondrial cardiolipin is required for Nlrp3 inflammasome activation', *Immunity*, 39(2), p. 311. doi: 10.1016/J.IMMUNI.2013.08.001.

Jackson, C. L., Walch, L. and Verbavatz, J. M. (2016) 'Lipids and Their Trafficking: An Integral Part of Cellular Organization', *Developmental cell*, 39(2), pp. 139–153. doi: 10.1016/J.DEVCEL.2016.09.030.

Jager, J. *et al.* (2007) 'Interleukin-1β-Induced Insulin Resistance in Adipocytes through Down-Regulation of Insulin Receptor Substrate-1 Expression', *Endocrinology*, 148(1), pp. 241–251. doi: 10.1210/EN.2006-0692.

Janeway, C. A. (1989) 'Approaching the asymptote? Evolution and revolution in immunology', *Cold Spring Harbor symposia on quantitative biology*, 54 Pt 1(1), pp.

1–13. doi: 10.1101/SQB.1989.054.01.003.

Janeway, C. A. and Medzhitov, R. (2002) ‘Innate immune recognition’, *Annual review of immunology*, 20, pp. 197–216. doi: 10.1146/ANNUREV.IMMUNOL.20.083001.084359.

Jin, T. *et al.* (2012) ‘Structures of the HIN Domain: DNA Complexes Reveal Ligand Binding and Activation Mechanisms of the AIM2 Inflammasome and IFI16 Receptor’, *Immunity*. doi: 10.1016/j.immuni.2012.02.014.

Jin, T. *et al.* (2013) ‘Structure of the absent in melanoma 2 (AIM2) pyrin domain provides insights into the mechanisms of AIM2 autoinhibition and inflammasome assembly’, *Journal of Biological Chemistry*, 288(19), pp. 13225–13235. doi: 10.1074/jbc.M113.468033.

Jo, E. K. *et al.* (2015) ‘Molecular mechanisms regulating NLRP3 inflammasome activation’, *Cellular & Molecular Immunology 2016 13:2*, 13(2), pp. 148–159. doi: 10.1038/cmi.2015.95.

Johansson, M. H. *et al.* (1997) ‘Natural killer cell tolerance in mice with mosaic expression of major histocompatibility complex class I transgene’, *The Journal of experimental medicine*, 186(3), pp. 353–364. doi: 10.1084/JEM.186.3.353.

Joshi, A. S. *et al.* (2012) ‘Cardiolipin and mitochondrial phosphatidylethanolamine have overlapping functions in mitochondrial fusion in Saccharomyces cerevisiae’, *The Journal of biological chemistry*, 287(21), pp. 17589–17597. doi: 10.1074/JBC.M111.330167.

Juliana, C. *et al.* (2012) ‘Non-transcriptional priming and deubiquitination regulate NLRP3 inflammasome activation’, *Journal of Biological Chemistry*. doi: 10.1074/jbc.M112.407130.

Kakimoto, P. A. *et al.* (2021) ‘Increased glycolysis is an early consequence of palmitate lipotoxicity mediated by redox signaling’, *Redox Biology*, 45, p. 102026. doi: 10.1016/J.REDOX.2021.102026.

Kang, P. B. *et al.* (2005) ‘The human macrophage mannose receptor directs Mycobacterium tuberculosis lipoarabinomannan-mediated phagosome biogenesis’, *The Journal of experimental medicine*, 202(7), pp. 987–999. doi: 10.1084/JEM.20051239.

Karasawa, T. *et al.* (2018a) ‘Saturated Fatty Acids Undergo Intracellular Crystallization and Activate the NLRP3 Inflammasome in Macrophages’, *Arteriosclerosis, Thrombosis, and Vascular Biology*, 38(4), pp. 744–756. doi: 10.1161/ATVBAHA.117.310581/-/DC1.

Karasawa, T. *et al.* (2018b) ‘Saturated Fatty Acids Undergo Intracellular Crystallization and Activate the NLRP3 Inflammasome in Macrophages’, *Arteriosclerosis, Thrombosis, and Vascular Biology*, 38(4), pp. 744–756. doi: 10.1161/ATVBAHA.117.310581/-/DC1.

Kary, S. and Burmester, G. (2003) ‘ANAKINRA: THE FIRST INTERLEUKIN-1 INHIBITOR IN THE TREATMENT OF RHEUMATOID ARTHRITIS’, *International Journal of Clinical Practice*, 57(3), pp. 231–234. doi: 10.1111/J.1742-1241.2003.TB10469.X.

Kayagaki, N. *et al.* (2011) 'Non-canonical inflammasome activation targets caspase-11', 479(7371), pp. 117–121. doi: 10.1038/nature10558.

Kayagaki, N. *et al.* (2015) 'Caspase-11 cleaves gasdermin D for non-canonical inflammasome signalling', 526(7575), pp. 666–671. Available at: https://pubmed.ncbi.nlm.nih.gov/26375259/ (Accessed: 2 May 2021).

Kelley, N. *et al.* (2019) 'The NLRP3 Inflammasome: An Overview of Mechanisms of Activation and Regulation', *International Journal of Molecular Sciences*, 20(13). doi: 10.3390/IJMS20133328.

Ketha, S. S., Singh, R. J. and Ketha, H. (2017) 'Role of Mass Spectrometry in Clinical Endocrinology', *Endocrinology and metabolism clinics of North America*, 46(3), pp. 593–613. doi: 10.1016/J.ECL.2017.04.001.

Klarić, L. *et al.* (2020) 'Glycosylation of immunoglobulin G is regulated by a large network of genes pleiotropic with inflammatory diseases', *Science Advances*. doi: 10.1126/sciadv.aax0301.

Koh, A. H. C. *et al.* (2020) 'What COVID-19 has taught us: lessons from around the globe', *Graefe's Archive for Clinical and Experimental Ophthalmology*, 258(10), p. 2091. doi: 10.1007/S00417-020-04791-9.

Kolczynska, K. *et al.* (2020) 'Diacylglycerol-evoked activation of PKC and PKD isoforms in regulation of glucose and lipid metabolism: a review', *Lipids in Health and Disease*, 19(1), p. 113. doi: 10.1186/S12944-020-01286-8.

Kolesnick, R. N. *et al.* (2000) 'Compartmentalization of Ceramide Signaling: Physical Foundations and Biological Effects', *Physiol*, 184, pp. 285–300. doi: 10.1002/1097-4652(200009)184:3.

Kong, S., Zhang, Y. H. and Zhang, W. (2018) 'Regulation of Intestinal Epithelial Cells Properties and Functions by Amino Acids', *BioMed Research International*, 2018, p. 2819154. doi: 10.1155/2018/2819154.

Korbecki, J. and Bajdak-Rusinek, K. (2019) 'The effect of palmitic acid on inflammatory response in macrophages: an overview of molecular mechanisms', *Inflammation Research*. Springer, pp. 915–932. doi: 10.1007/s00011-019-01273-5.

Kraaij, M. D. *et al.* (2014) 'Human monocytes produce interferon-gamma upon stimulation with LPS', *Cytokine*, 67(1), pp. 7–12. doi: 10.1016/J.CYTO.2014.02.001.

Kridel, S. J. *et al.* (2004) 'Orlistat Is a Novel Inhibitor of Fatty Acid Synthase with Antitumor Activity', *Cancer Research*, 64(6), pp. 2070–2075. doi: 10.1158/0008-5472.CAN-03-3645.

Krishnan, S. M. *et al.* (2016) 'Inflammasome activity is essential for one kidney/deoxycorticosterone acetate/salt-induced hypertension in mice', *British journal of pharmacology*, 173(4), pp. 752–765. doi: 10.1111/BPH.13230.

Kuhajda, F. P. *et al.* (2000) 'Synthesis and antitumor activity of an inhibitor of fatty acid synthase', *Proceedings of the National Academy of Sciences of the United States of America*, 97(7), p. 3450. doi: 10.1073/PNAS.050582897.

Kumar, A. *et al.* (2020) 'Role of pyruvate kinase M2 in oxidized LDL-induced macrophage foam cell formation and inflammation', *Journal of Lipid Research*, 61(3),

pp. 351–364. doi: 10.1194/jlr.RA119000382.

Kumar, B. S. (2023) ‘Desorption electrospray ionization mass spectrometry imaging (DESI-MSI) in disease diagnosis: an overview’, *Analytical Methods*, 15(31), pp. 3768–3784. doi: 10.1039/D3AY00867C.

Kumari, P. *et al.* (2020a) ‘AIM2 in health and disease: inflammasome and beyond’, *Immunological reviews*, 297(1), p. 83. doi: 10.1111/IMR.12903.

Kumari, P. *et al.* (2020b) ‘AIM2 in health and disease: inflammasome and beyond’, *Immunological reviews*, 297(1), p. 83. doi: 10.1111/IMR.12903.

Kumari, P. *et al.* (2020c) ‘AIM2 in health and disease: Inflammasome and beyond’, *Immunological Reviews*, 297(1), pp. 83–95. doi: 10.1111/IMR.12903.

Kwon, H. J. *et al.* (2009) ‘Structure of N-Terminal Domain of NPC1 Reveals Distinct Subdomains for Binding and Transfer of Cholesterol’, *Cell*. doi: 10.1016/j.cell.2009.03.049.

L’homme, L. *et al.* (2013) ‘Unsaturated fatty acids prevent activation of NLRP3 inflammasome in human monocytes/macrophages’, *Journal of Lipid Research*, 54(11). Available at: http://www.jlr.org/article/S0022227520350513/fulltext (Accessed: 28 October 2024).

de la Roche, M. *et al.* (2018) ‘Trafficking of cholesterol to the ER is required for NLRP3 inflammasome activation’, *Journal of Cell Biology*, 217(10), pp. 3560–3576. doi: 10.1083/JCB.201709057.

Lacy, P. (2006) ‘Mechanisms of degranulation in neutrophils’, *Allergy, asthma, and clinical immunology : official journal of the Canadian Society of Allergy and Clinical Immunology*, 2(3). doi: 10.1186/1710-1492-2-3-98.

Ladygina, N., Martin, B. R. and Altman, A. (2011) ‘Dynamic Palmitoylation and the Role of DHHC Proteins in T Cell Activation and Anergy’, *Advances in immunology*, 109, p. 1. doi: 10.1016/B978-0-12-387664-5.00001-7.

Lai, P. *et al.* (2023) ‘Current hotspot and study trend of innate immunity in COVID-19: a bibliometric analysis from 2020 to 2022’, *Frontiers in Immunology*, 14, p. 1135334. doi: 10.3389/FIMMU.2023.1135334/BIBTEX.

Lam, N., Lee, Y. S. and Farber, D. L. (2024) ‘A guide to adaptive immune memory’, *Nature Reviews Immunology 2024*, pp. 1–20. doi: 10.1038/s41577-024-01040-6.

Lammert, C. R. *et al.* (2020) ‘AIM2 inflammasome surveillance of DNA damage shapes neurodevelopment’, *Nature*, 580(7805), p. 647. doi: 10.1038/S41586-020-2174-3.

Langston, P. K., Shibata, M. and Horng, T. (2017) ‘Metabolism supports macrophage activation’, *Frontiers in Immunology*, 8(JAN), p. 245913. doi: 10.3389/FIMMU.2017.00061/BIBTEX.

Lee *et al.* (2023) ‘Disruptions in endocytic traffic contribute to the activation of the NLRP3 inflammasome’, *Science Signaling*, 16(773). doi: 10.1126/scisignal.abm7134.

Lee, G. S. *et al.* (2012) *The calcium-sensing receptor regulates the NLRP3*

inflammasome through Ca2+ and cAMP, *Nature*. Nature. Available at: https://pubmed.ncbi.nlm.nih.gov/23143333/ (Accessed: 28 October 2024).

Lee, H. J. *et al.* (2020) 'Formation and Maturation of the Phagosome: A Key Mechanism in Innate Immunity against Intracellular Bacterial Infection', *Microorganisms*, 8(9), p. 1298. doi: 10.3390/MICROORGANISMS8091298.

Lee, J. M. *et al.* (2023) 'Control of protein stability by post-translational modifications', *Nature Communications 2023 14:1*, 14(1), pp. 1–16. doi: 10.1038/s41467-023-35795-8.

Lee, W. L., Harrison, R. E. and Grinstein, S. (2003) 'Phagocytosis by neutrophils', *Microbes and Infection*, 5(14), pp. 1299–1306. doi: 10.1016/j.micinf.2003.09.014.

Leemans, J. C., Cassel, S. L. and Sutterwala, F. S. (2011) 'Sensing damage by the NLRP3 inflammasome', *Immunological reviews*, 243(1), p. 152. doi: 10.1111/J.1600-065X.2011.01043.X.

Leishman, S. *et al.* (2024) 'Fatty acid synthesis promotes inflammasome activation through NLRP3 palmitoylation', *Cell Reports*, 43(8), p. 114516. doi: 10.1016/J.CELREP.2024.114516.

Leishman, S., Aljadeed, N. M. and Anand, P. K. (2024) 'Protocol for a semi-quantitative approach to identify protein S-palmitoylation in cultured cells by acyl biotin exchange assay', *STAR protocols*, 5(2). doi: 10.1016/J.XPRO.2024.103054.

Leong, X. F. (2021) 'Lipid Oxidation Products on Inflammation-Mediated Hypertension and Atherosclerosis: A Mini Review', *Frontiers in Nutrition*, 8, p. 717740. doi: 10.3389/FNUT.2021.717740/BIBTEX.

Lerner, A. G. *et al.* (2012) 'IRE1α induces thioredoxin-interacting protein to activate the NLRP3 inflammasome and promote programmed cell death under irremediable ER stress', *Cell metabolism*, 16(2), pp. 250–264. doi: 10.1016/J.CMET.2012.07.007.

Leroux, A. *et al.* (2012) 'Toxic lipids stored by Kupffer cells correlates with their pro-inflammatory phenotype at an early stage of steatohepatitis', *Journal of hepatology*, 57(1), pp. 141–149. doi: 10.1016/J.JHEP.2012.02.028.

Levental, I. *et al.* (2010) 'Palmitoylation regulates raft affinity for the majority of integral raft proteins', *Proceedings of the National Academy of Sciences of the United States of America*, 107(51), pp. 22050–22054. doi: 10.1073/PNAS.1016184107.

Li, C. *et al.* (2020) 'Crosstalk Between Acid Sphingomyelinase and Inflammasome Signaling and Their Emerging Roles in Tissue Injury and Fibrosis', *Frontiers in Cell and Developmental Biology*, 7, p. 503855. doi: 10.3389/FCELL.2019.00378/BIBTEX.

Li, D. and Wu, M. (2021) 'Pattern recognition receptors in health and diseases', *Signal Transduction and Targeted Therapy*. Nature Publishing Group, pp. 1–24. doi: 10.1038/s41392-021-00687-0.

Li, P. and Bartlett, M. G. (2014) 'A review of sample preparation methods for quantitation of small-molecule analytes in brain tissue by liquid chromatography tandem mass spectrometry (LC-MS/MS)', *Analytical Methods*, 6(16), pp. 6183–6207. doi: 10.1039/C4AY00915K.

Li, S. *et al.* (2018) ‘Identification of ethyl pyruvate as a NLRP3 inflammasome inhibitor that preserves mitochondrial integrity’, *Molecular medicine (Cambridge, Mass.)*, 24(1). doi: 10.1186/S10020-018-0006-9.

Li, X. *et al.* (2017) ‘MARK4 regulates NLRP3 positioning and inflammasome activation through a microtubule-dependent mechanism’, *Nature Communications*, 8, p. 15986. doi: 10.1038/NCOMMS15986.

Li, X. *et al.* (2021) ‘Macrophage HIF-2α suppresses NLRP3 inflammasome activation and alleviates insulin resistance’, *Cell Reports*, 36(8), p. 109607. doi: 10.1016/J.CELREP.2021.109607.

Li, X. *et al.* (2023) ‘Inflammation and aging: signaling pathways and intervention therapies’, *Signal Transduction and Targeted Therapy 2023 8:1*, 8(1), pp. 1–29. doi: 10.1038/s41392-023-01502-8.

Li, Y. *et al.* (2013) ‘Activation of Sterol Regulatory Element Binding Protein and NLRP3 Inflammasome in Atherosclerotic Lesion Development in Diabetic Pigs’, *PLoS ONE*, 8(6). doi: 10.1371/journal.pone.0067532.

Lichtnekert, J. *et al.* (2013) ‘Changes in macrophage phenotype as the immune response evolves’, *Current opinion in pharmacology*, 13(4), p. 555. doi: 10.1016/J.COPH.2013.05.013.

Lietz, C. B., Gemperline, E. and Li, L. (2013) ‘Qualitative and quantitative mass spectrometry imaging of drugs and metabolites’, *Advanced drug delivery reviews*, 65(8), p. 1074. doi: 10.1016/J.ADDR.2013.04.009.

Linder, M. E. and Deschenes, R. J. (2007a) ‘Palmitoylation: policing protein stability and traffic’, *Nature Reviews Molecular Cell Biology 2007 8:1*, 8(1), pp. 74–84. doi: 10.1038/nrm2084.

Linder, M. E. and Deschenes, R. J. (2007b) ‘Palmitoylation: policing protein stability and traffic’, *Nature Reviews Molecular Cell Biology 2007 8:1*, 8(1), pp. 74–84. doi: 10.1038/nrm2084.

Lindsey, T. L. *et al.* (2008) ‘Virulence determinants from a cystic fibrosis isolate of Pseudomonas aeruginosa include isocitrate lyase’, *Microbiology*, 154(6), pp. 1616–1627. doi: 10.1099/MIC.0.2007/014506-0.

Listenberger, L. L. *et al.* (2003) ‘Triglyceride accumulation protects against fatty acid-induced lipotoxicity’, *Proceedings of the National Academy of Sciences of the United States of America*, 100(6), pp. 3077–3082. doi: 10.1073/PNAS.0630588100.

Liu. *et al.* (2022) ‘Ceramide induces pyroptosis through TXNIP/NLRP3/GSDMD pathway in HUVECs’, *BMC Molecular and Cell Biology*, 23(1). doi: 10.1186/s12860-022-00459-w.

Liu, K. *et al.* (2002) ‘Immune Tolerance After Delivery of Dying Cells to Dendritic Cells In Situ’, *The Journal of Experimental Medicine*, 196(8), p. 1091. doi: 10.1084/JEM.20021215.

Liu, T. *et al.* (2017) ‘NF-κB signaling in inflammation’, *Signal Transduction and Targeted Therapy*, 2, p. 17023. doi: 10.1038/SIGTRANS.2017.23.

Liu, W. *et al.* (2020) ‘The Ubiquitin Conjugating Enzyme: An Important Ubiquitin

Transfer Platform in Ubiquitin-Proteasome System', *International Journal of Molecular Sciences*, 21(8). doi: 10.3390/IJMS21082894.

Liu, X. *et al.* (2016a) 'Inflammasome-activated gasdermin D causes pyroptosis by forming membrane pores', *Nature*, 535(7610), pp. 153–158. doi: 10.1038/NATURE18629.

Liu, X. *et al.* (2016b) 'Inflammasome - activated gasdermin D causes pyroptosis by forming membrane pores', *Nature*, 535(7610), p. 153. doi: 10.1038/NATURE18629.

Liu, Zhuang *et al.* (2022) 'Emerging roles of protein palmitoylation and its modifying enzymes in cancer cell signal transduction and cancer therapy', *International Journal of Biological Sciences*, 18(8), p. 3447. doi: 10.7150/IJBS.72244.

Lopez-Castejon, G. *et al.* (2013) 'Deubiquitinases regulate the activity of caspase-1 and interleukin-1β secretion via assembly of the inflammasome', *Journal of Biological Chemistry*. doi: 10.1074/jbc.M112.422238.

Lordén, G. *et al.* (2017) 'Lipin-2 regulates NLRP3 inflammasome by affecting P2X7 receptor activation', *The Journal of Experimental Medicine*, 214(2), p. 511. doi: 10.1084/JEM.20161452.

Lu, Y. *et al.* (2019) '2-Bromopalmitate targets retinoic acid receptor alpha and overcomes all-trans retinoic acid resistance of acute promyelocytic leukemia', *Haematologica*, 104(1), p. 102. doi: 10.3324/HAEMATOL.2018.191916.

Luheshi, N. M. *et al.* (2012) 'Sphingosine regulates the NLRP3-inflammasome and IL-1β release from macrophages', *European Journal of Immunology*, 42(3), pp. 716–725. doi: 10.1002/EJI.201142079.

Macklin, A., Khan, S. and Kislinger, T. (2020) 'Recent advances in mass spectrometry based clinical proteomics: Applications to cancer research', *Clinical Proteomics*. BMC. doi: 10.1186/s12014-020-09283-w.

Maedler, K. *et al.* (2002) 'Glucose-induced β cell production of IL-1β contributes to glucotoxicity in human pancreatic islets', *The Journal of Clinical Investigation*, 110(6), pp. 851–860. doi: 10.1172/JCI15318.

Magupalli, V. G. *et al.* (2020) 'HDAC6 mediates an aggresome-like mechanism for NLRP3 and pyrin inflammasome activation', *Science*, 369(6509). doi: 10.1126/SCIENCE.AAS8995/SUPPL_FILE/AAS8995-MAGUPALLI-SM.PDF.

Maier, T., Leibundgut, M. and Ban, N. (2008) 'The crystal structure of a mammalian fatty acid synthase', 321(5894), pp. 1315–1322. Available at: https://pubmed.ncbi.nlm.nih.gov/18772430/ (Accessed: 17 July 2024).

Majeed, S. *et al.* (2021) 'Lipid Membrane Mimetics in Functional and Structural Studies of Integral Membrane Proteins', *Membranes*, 11(9), p. 685. doi: 10.3390/MEMBRANES11090685.

Malech, H. L., Deleo, F. R. and Quinn, M. T. (2014) 'The Role of Neutrophils in the Immune System: An Overview', *Methods in molecular biology (Clifton, N.J.)*, 1124, p. 3. doi: 10.1007/978-1-62703-845-4_1.

Mangan, M. S. J. *et al.* (2018) 'Targeting the NLRP3 inflammasome in inflammatory diseases', *Nature Reviews Drug Discovery 2018 17:8*, 17(8), pp. 588–606. doi:

10.1038/nrd.2018.97.

Manh, T. P. V. *et al.* (2013) 'Plasmacytoid, conventional, and monocyte-derived dendritic cells undergo a profound and convergent genetic reprogramming during their maturation', *European Journal of Immunology*, 43(7), p. 1706. doi: 10.1002/EJI.201243106.

Margraf, A. and Perretti, M. (2022) 'Immune Cell Plasticity in Inflammation: Insights into Description and Regulation of Immune Cell Phenotypes', *Cells*, 11(11), p. 1824. doi: 10.3390/CELLS11111824.

Mariathasan, S. *et al.* (2006) 'Cryopyrin activates the inflammasome in response to toxins and ATP', *Nature*, 440(7081), pp. 228–232. doi: 10.1038/NATURE04515.

Marshall, J. S. *et al.* (2018) 'An introduction to immunology and immunopathology', *Allergy, Asthma and Clinical Immunology*, 14(2), pp. 1–10. doi: 10.1186/S13223-018-0278-1/TABLES/4.

Martin, S. and Parton, R. G. (2006) 'Lipid droplets: a unified view of a dynamic organelle', *Nature reviews. Molecular cell biology*, 7(5), pp. 373–378. doi: 10.1038/NRM1912.

Martínez-Ruiz, A. and Lamas, S. (2004) 'S-nitrosylation: A potential new paradigm in signal transduction', *Cardiovascular Research*, 62(1), pp. 43–52. doi: 10.1016/J.CARDIORES.2004.01.013/2/62-1-43-FIG2.GIF.

Martinon, F., Burns, K. and Tschopp, J. (2002a) 'The inflammasome: a molecular platform triggering activation of inflammatory caspases and processing of proIL-beta', *Molecular cell*, 10(2), pp. 417–426. doi: 10.1016/S1097-2765(02)00599-3.

Martinon, F., Burns, K. and Tschopp, J. (2002b) 'The Inflammasome: A molecular platform triggering activation of inflammatory caspases and processing of proIL-β', *Molecular Cell*, 10(2), pp. 417–426. doi: 10.1016/S1097-2765(02)00599-3.

Martinon, F. and Tschopp, J. (2004) 'Inflammatory caspases: Linking an intracellular innate immune system to autoinflammatory diseases', *Cell*, 117(5), pp. 561–574. doi: 10.1016/j.cell.2004.05.004.

Mass, E. *et al.* (2023) 'Tissue-specific macrophages: how they develop and choreograph tissue biology', *Nature Reviews Immunology 2023 23:9*, 23(9), pp. 563–579. doi: 10.1038/s41577-023-00848-y.

Matico, R. E. *et al.* (2024) 'Structural basis of the human NAIP/NLRC4 inflammasome assembly and pathogen sensing', *Nature Structural & Molecular Biology 2024 31:1*, 31(1), pp. 82–91. doi: 10.1038/s41594-023-01143-z.

Matsuda, M. *et al.* (2001) 'SREBP cleavage-activating protein (SCAP) is required for increased lipid synthesis in liver induced by cholesterol deprivation and insulin elevation', *Genes & Development*, 15(10), p. 1206. doi: 10.1101/GAD.891301.

Matzinger, P. (2002) 'The danger model: a renewed sense of self', *Science (New York, N.Y.)*, 296(5566), pp. 301–305. doi: 10.1126/SCIENCE.1071059.

Maxfield, F. R., Barbosa-Lorenzi, V. C. and Singh, R. K. (2019) 'Digestive Exophagy: Phagocyte digestion of objects too large for phagocytosis', *Traffic (Copenhagen, Denmark)*, 21(1), p. 6. doi: 10.1111/TRA.12712.

Maxfield, F. R. and van Meer, G. (2010) 'Cholesterol, the central lipid of mammalian cells', *Current Opinion in Cell Biology*. doi: 10.1016/j.ceb.2010.05.004.

McKinney, J. D. *et al.* (2000) 'Persistence of Mycobacterium tuberculosis in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase', *Nature*, 406(6797), pp. 735–738. doi: 10.1038/35021074.

McLean, M. H. and El-Omar, E. M. (2009) 'Genetic aspects of inflammation', *Current Opinion in Pharmacology*, 9(4), pp. 370–374. doi: 10.1016/J.COPH.2009.06.003.

Medzhitov, R., Preston-Hurlburt, P. and Janeway, C. A. (1997) 'A human homologue of the Drosophila Toll protein signals activation of adaptive immunity', *Nature*, 388(6640), pp. 394–397. doi: 10.1038/41131.

Meissner, F., Molawi, K. and Zychlinsky, A. (2008) 'Superoxide dismutase 1 regulates caspase-1 and endotoxic shock', *Nature immunology*, 9(8), pp. 866–872. doi: 10.1038/NI.1633.

Meluh, P. B. and Koshland, D. (1995) 'Evidence that the MIF2 gene of Saccharomyces cerevisiae encodes a centromere protein with homology to the mammalian centromere protein CENP-C', *Molecular biology of the cell*, 6(7), pp. 793–807. doi: 10.1091/MBC.6.7.793.

Menendez, J. A. *et al.* (2024) 'Fatty acid synthase (FASN) signalome: A molecular guide for precision oncology', *Molecular Oncology*, 18(3), p. 479. doi: 10.1002/1878-0261.13582.

de Menthière, C. S. *et al.* (2003) 'INFEVERS: the Registry for FMF and hereditary inflammatory disorders mutations', *Nucleic acids research*, 31(1), pp. 282–285. doi: 10.1093/NAR/GKG031.

Mera, P. *et al.* (2009) 'C75 is converted to C75-CoA in the hypothalamus, where it inhibits carnitine palmitoyltransferase 1 and decreases food intake and body weight', *Biochemical Pharmacology*, 77(6), pp. 1084–1095. doi: 10.1016/J.BCP.2008.11.020.

Merad, M. *et al.* (2013) 'The Dendritic Cell Lineage: Ontogeny and Function of Dendritic Cells and Their Subsets in the Steady State and the Inflamed Setting', *Annual review of immunology*, 31, pp. 10.1146/annurev-immunol-020711–074950. doi: 10.1146/ANNUREV-IMMUNOL-020711-074950.

Mesquita, I. *et al.* (2020) 'The Absence of HIF-1α Increases Susceptibility to Leishmania donovani Infection via Activation of BNIP3/mTOR/SREBP-1c Axis', *Cell Reports*, 30(12), pp. 4052-4064.e7. doi: 10.1016/j.celrep.2020.02.098.

Mia, M. M., Boersema, M. and Bank, R. A. (2014) 'Interleukin-1β attenuates myofibroblast formation and extracellular matrix production in dermal and lung fibroblasts exposed to transforming growth factor-β1', *PLoS ONE*, 9(3), p. 91559. doi: 10.1371/journal.pone.0091559.

Miao, R. *et al.* (2023) 'Gasdermin D permeabilization of mitochondrial inner and outer membranes accelerates and enhances pyroptosis', *Immunity*, 56(11), pp. 2523-2541.e8. doi: 10.1016/J.IMMUNI.2023.10.004.

Mills, E. L. *et al.* (2016) 'Succinate Dehydrogenase Supports Metabolic Repurposing of Mitochondria to Drive Inflammatory Macrophages', *Cell*, 167(2), pp. 457-470.e13. doi: 10.1016/J.CELL.2016.08.064.

Mills, E. L. *et al.* (2018) ‘Itaconate is an anti-inflammatory metabolite that activates Nrf2 via alkylation of KEAP1’, *Nature 2018 556:7699*, 556(7699), pp. 113–117. doi: 10.1038/nature25986.

Minai-Fleminger, Y. and Levi-Schaffer, F. (2009) ‘Mast cells and eosinophils: The two key effector cells in allergic inflammation’, *Inflammation Research*, 58(10), pp. 631–638. doi: 10.1007/S00011-009-0042-6/FIGURES/2.

Minkah, N. K. *et al.* (2019) ‘Innate immunity limits protective adaptive immune responses against pre-erythrocytic malaria parasites’, *Nature Communications 2019 10:1*, 10(1), pp. 1–14. doi: 10.1038/s41467-019-11819-0.

Misawa, T. *et al.* (2013) ‘Microtubule-driven spatial arrangement of mitochondria promotes activation of the NLRP3 inflammasome’, 14(5), pp. 454–460. doi: 10.1038/ni.2550.

Mishra, B. B. *et al.* (2013) ‘Nitric oxide controls the immunopathology of tuberculosis by inhibiting NLRP3 inflammasome-dependent processing of IL-1β’, *Nature immunology*, 14(1), pp. 52–60. doi: 10.1038/NI.2474.

Mitchell, A. J., Roediger, B. and Weninger, W. (2014) ‘Monocyte homeostasis and the plasticity of inflammatory monocytes’, *Cellular Immunology*, 291(1–2), pp. 22–31. doi: 10.1016/J.CELLIMM.2014.05.010.

Mitchell, D. A. *et al.* (2006) ‘Protein palmitoylation by a family of DHHC protein S-acyltransferases’, *Journal of Lipid Research*, 47(6), pp. 1118–1127. doi: 10.1194/JLR.R600007-JLR200/ASSET/6D097F22-CE52-4A18-9D9E-C230586BBAC6/MAIN.ASSETS/GR1.JPG.

Moche, M. *et al.* (1999) ‘Structure of the complex between the antibiotic cerulenin and its target, β-ketoacyl-acyl carrier protein synthase’, *Journal of Biological Chemistry*, 274(10), pp. 6031–6034. doi: 10.1074/jbc.274.10.6031.

Molla, M. D. *et al.* (2020) ‘Role of Caspase-1 in the Pathogenesis of Inflammatory-Associated Chronic Noncommunicable Diseases’, *Journal of Inflammation Research*, 13, p. 749. doi: 10.2147/JIR.S277457.

Moltrasio, C., Romagnuolo, M. and Marzano, A. V. (2022) ‘NLRP3 inflammasome and NLRP3-related autoinflammatory diseases: From cryopyrin function to targeted therapies’, *Frontiers in Immunology*, 13. doi: 10.3389/FIMMU.2022.1007705.

Morales, J. *et al.* (1998) ‘Plasma membrane localization of G alpha z requires two signals’, *Molecular biology of the cell*, 9(1), pp. 1–14. doi: 10.1091/MBC.9.1.1.

Morita, S. Y. and Terada, T. (2015) ‘Enzymatic measurement of phosphatidylglycerol and cardiolipin in cultured cells and mitochondria’, *Scientific reports*, 5. doi: 10.1038/SREP11737.

Morris, K. N. and Mitchell, A. M. (2023) ‘Phosphatidylglycerol Is the Lipid Donor for Synthesis of Phospholipid-Linked Enterobacterial Common Antigen’, *Journal of Bacteriology*, 205(1), pp. e00403-22. doi: 10.1128/JB.00403-22.

Mortimer, L. *et al.* (2016) ‘NLRP3 inflammasome inhibition is disrupted in a group of auto-inflammatory disease CAPS mutations’, 17(10), pp. 1176–1186. Available at: https://pubmed.ncbi.nlm.nih.gov/27548431/ (Accessed: 13 March 2023).

Muñoz-Planillo, R. *et al.* (2013a) 'K+ Efflux Is the Common Trigger of NLRP3 Inflammasome Activation by Bacterial Toxins and Particulate Matter', *Immunity*, 38(6), pp. 1142–1153. doi: 10.1016/j.immuni.2013.05.016.

Muñoz-Planillo, R. *et al.* (2013b) 'K+ Efflux Is the Common Trigger of NLRP3 Inflammasome Activation by Bacterial Toxins and Particulate Matter', *Immunity*, 38(6), pp. 1142–1153. doi: 10.1016/J.IMMUNI.2013.05.016.

Muñoz-Planillo, R. *et al.* (2013c) 'K^+ efflux is the common trigger of NLRP3 inflammasome activation by bacterial toxins and particulate matter', *Immunity*, 38(6), pp. 1142–1153. doi: 10.1016/J.IMMUNI.2013.05.016.

Munro, S. (2003) 'Lipid Rafts: Elusive or Illusive?', *Cell*, 115(4), pp. 377–388. doi: 10.1016/S0092-8674(03)00882-1.

Murakami, T. *et al.* (2012) 'Critical role for calcium mobilization in activation of the NLRP3 inflammasome', *Proceedings of the National Academy of Sciences*. doi: 10.1073/pnas.1117765109.

Murakami, Tomohiko *et al.* (2012) 'Critical role for calcium mobilization in activation of the NLRP3 inflammasome', *Proceedings of the National Academy of Sciences of the United States of America*, 109(28), pp. 11282–11287. doi: 10.1073/PNAS.1117765109/-/DCSUPPLEMENTAL.

Murphy, R. C. (2018) 'Challenges in mass spectrometry-based lipidomics of neutral lipids', *TrAC - Trends in Analytical Chemistry*, 107, pp. 91–98. doi: 10.1016/j.trac.2018.07.023.

Murray, P. J. *et al.* (2014) 'Macrophage activation and polarization: nomenclature and experimental guidelines', *Immunity*, 41(1), pp. 14–20. doi: 10.1016/J.IMMUNI.2014.06.008.

Murru, E. *et al.* (2022) 'Impact of Dietary Palmitic Acid on Lipid Metabolism', 9, p. 861664. doi: 10.3389/FNUT.2022.861664.

Mushegian, A. and Medzhitov, R. (2001) 'Evolutionary perspective on innate immune recognition', *The Journal of Cell Biology*, 155(5), p. 705. doi: 10.1083/JCB.200107040.

Nagae, M. and Yamaguchi, Y. (2020) 'Structural Aspects of Carbohydrate Recognition Mechanisms of C-Type Lectins', *Current Topics in Microbiology and Immunology*, 429, pp. 147–176. doi: 10.1007/82_2019_181/FIGURES/7.

Nakahira, K. *et al.* (2011) 'Autophagy proteins regulate innate immune responses by inhibiting the release of mitochondrial DNA mediated by the NALP3 inflammasome', *Nature immunology*, 12(3), pp. 222–230. doi: 10.1038/NI.1980.

Natesan, V. and Kim, S. J. (2021) 'Lipid Metabolism, Disorders and Therapeutic Drugs – Review', *Biomolecules & Therapeutics*, 29(6), p. 596. doi: 10.4062/BIOMOLTHER.2021.122.

Nelson, D. L. and Cox, M. M. (2008) *Lehninger Principles of Biochemistry, Second Edition*, *W H Freeman*. W. H. Freeman. Available at: https://books.google.com/books/about/Lehninger_Principles_of_Biochemistry.html?id=5Ek9J4p3NfkC (Accessed: 9 December 2024).

Netea, M. G. *et al.* (2020) ‘Defining trained immunity and its role in health and disease’, *Nature Reviews Immunology 2020 20:6*, 20(6), pp. 375–388. doi: 10.1038/s41577-020-0285-6.

Netea, M. G., Quintin, J. and Van Der Meer, J. W. M. (2011) ‘Perspective Trained Immunity: A Memory for Innate Host Defense’. doi: 10.1016/j.chom.2011.04.006.

Nguyen, C. *et al.* (2014) ‘Trapping the dynamic acyl carrier protein in fatty acid biosynthesis’, *Nature*, 505(7483), p. 427. doi: 10.1038/NATURE12810.

Nguyen, M. T. A. *et al.* (2007) ‘A subpopulation of macrophages infiltrates hypertrophic adipose tissue and is activated by free fatty acids via toll-like receptors 2 and 4 and JNK-dependent pathways’, *Journal of Biological Chemistry*, 282(48), pp. 35279–35292. doi: 10.1074/jbc.M706762200.

Ning, W. *et al.* (2021) ‘GPS-Palm: a deep learning-based graphic presentation system for the prediction of S-palmitoylation sites in proteins’, *Briefings in Bioinformatics*, 22(2), pp. 1836–1847. doi: 10.1093/BIB/BBAA038.

Niu, T. *et al.* (2021) ‘NLRP3 phosphorylation in its LRR domain critically regulates inflammasome assembly’, 12(1), pp. 1–15. Available at: /pmc/articles/PMC8494922/ (Accessed: 12 June 2024).

Nordenfelt, P. and Tapper, H. (2011) ‘Phagosome dynamics during phagocytosis by neutrophils’, *Journal of leukocyte biology*, 90(2), pp. 271–284. doi: 10.1189/JLB.0810457.

Okumura, R. and Takeda, K. (2017) ‘Roles of intestinal epithelial cells in the maintenance of gut homeostasis’, *Experimental & Molecular Medicine 2017 49:5*, 49(5), pp. e338–e338. doi: 10.1038/emm.2017.20.

Olona, A., Leishman, S. and Anand, P. K. (2022) ‘The NLRP3 inflammasome: regulation by metabolic signals’, *Trends in Immunology*, 43(12), pp. 978–989. doi: 10.1016/j.it.2022.10.003.

Olshina, M. A. and Sharon, M. (2016) ‘Mass Spectrometry: A Technique of Many Faces’, *Quarterly reviews of biophysics*, 49, p. e18. doi: 10.1017/S0033583516000160.

Ortega, M. A. *et al.* (2023) ‘Reframing the link between metabolism and NLRP3 inflammasome: therapeutic opportunities’, *Frontiers in Immunology*, 14, p. 1232629. doi: 10.3389/FIMMU.2023.1232629.

Ouyang, H. *et al.* (2023) ‘Mechanisms of pulmonary microvascular endothelial cells barrier dysfunction induced by LPS: The roles of ceramides and the Txnip/NLRP3 inflammasome’, *Microvascular Research*, 147, p. 104491. doi: 10.1016/J.MVR.2023.104491.

Palazón-Riquelme, P. *et al.* (2018) ‘USP7 and USP47 deubiquitinases regulate NLRP3 inflammasome activation’, *EMBO reports*, 19(10). doi: 10.15252/EMBR.201744766/SUPPL_FILE/EMBR201744766-SUP-0009-SDATAEVFIG.ZIP.

Petan, T. and Manček-Keber, M. (2022) ‘Half is enough: Oxidized lysophospholipids as novel bioactive molecules’, *Free Radical Biology and Medicine*, 188, pp. 351–362. doi: 10.1016/J.FREERADBIOMED.2022.06.228.

Piccini, A. *et al.* (2008) 'ATP is released by monocytes stimulated with pathogen-sensing receptor ligands and induces IL-1 and IL-18 secretion in an autocrine way', *Proceedings of the National Academy of Sciences*. doi: 10.1073/pnas.0709684105.

Pierini, R. *et al.* (2012) 'AIM2/ASC triggers caspase-8-dependent apoptosis in Francisella-infected caspase-1-deficient macrophages', *Cell Death and Differentiation*, 19(10), p. 1709. doi: 10.1038/CDD.2012.51.

Pilla, D. M. *et al.* (2014) 'Guanylate binding proteins promote caspase-11-dependent pyroptosis in response to cytoplasmic LPS', *Proceedings of the National Academy of Sciences of the United States of America*, 111(16), pp. 6046–6051. doi: 10.1073/PNAS.1321700111.

Prasse, A. *et al.* (2007) 'IL-10-producing monocytes differentiate to alternatively activated macrophages and are increased in atopic patients', *The Journal of allergy and clinical immunology*, 119(2), pp. 464–471. doi: 10.1016/J.JACI.2006.09.030.

Prince, L. R. *et al.* (2004) 'The Role of Interleukin-1β in Direct and Toll-Like Receptor 4-Mediated Neutrophil Activation and Survival', *The American Journal of Pathology*, 165(5), p. 1819. doi: 10.1016/S0002-9440(10)63437-2.

Pugazhenthi, S. *et al.* (2013) 'Induction of an Inflammatory Loop by Interleukin-1b and Tumor Necrosis Factor-a Involves NF-kB and STAT-1 in Differentiated Human Neuroprogenitor Cells) Induction of an Inflammatory Loop by Interleukin-1b and Tumor Necrosis Factor-a Involves NF-kB and STAT-1 in Differentiated Human Neuroprogenitor Cells', *PLoS ONE*, 8(7), p. 69585. doi: 10.1371/journal.pone.0069585.

Putilina, T., Wong, P. and Gentleman, S. (1999) 'The DHHC domain: a new highly conserved cysteine-rich motif', *Molecular and cellular biochemistry*, 195(1–2), pp. 219–226. doi: 10.1023/A:1006932522197.

Py, B. F. *et al.* (2013) 'Deubiquitination of NLRP3 by BRCC3 Critically Regulates Inflammasome Activity', *Molecular Cell*, 49(2), pp. 331–338. doi: 10.1016/j.molcel.2012.11.009.

Qian, C. *et al.* (2019) 'Heterogeneous macrophages: Supersensors of exogenous inducing factors', *Scandinavian Journal of Immunology*, 90(1), p. e12768. doi: 10.1111/SJI.12768.

Qin, Y. *et al.* (2021) 'TRIM28 SUMOylates and stabilizes NLRP3 to facilitate inflammasome activation', *Nature communications*, 12(1). doi: 10.1038/S41467-021-25033-4.

Qiu, Y. *et al.* (2023) 'A Lipid Perspective on Regulated Pyroptosis', *International Journal of Biological Sciences*, 19(8), p. 2333. doi: 10.7150/IJBS.81017.

Que, X. *et al.* (2024) 'Fantastic voyage: The journey of NLRP3 inflammasome activation', *Genes & Diseases*, 11(2), p. 819. doi: 10.1016/J.GENDIS.2023.01.009.

Qureshi, S. T. *et al.* (1999) 'Endotoxin-tolerant mice have mutations in toll-like receptor 4 (Tlr4)', *Journal of Experimental Medicine*, 189(4), pp. 615–625. doi: 10.1084/jem.189.4.615.

Rajaṁaki, K. *et al.* (2010) 'Cholesterol Crystals Activate the NLRP3 Inflammasome in Human Macrophages: A Novel Link between Cholesterol Metabolism and

Inflammation', *PLoS ONE*, 5(7). doi: 10.1371/JOURNAL.PONE.0011765.

Ramamurthy, D. *et al.* (2021) 'Recent advances in immunotherapies against infectious diseases', *Immunotherapy Advances*, 1(1), pp. 1–16. doi: 10.1093/IMMADV/LTAA007.

Ramazi, S. and Zahiri, J. (2021) 'Post-translational modifications in proteins: resources, tools and prediction methods', *Database: The Journal of Biological Databases and Curation*, 2021. doi: 10.1093/DATABASE/BAAB012.

Ramírez, J. and Cañete, J. D. (2018) 'Anakinra for the treatment of rheumatoid arthritis: a safety evaluation', *Expert opinion on drug safety*, 17(7), pp. 727–732. doi: 10.1080/14740338.2018.1486819.

Ramos, H. J. and Gale, M. (2011) 'RIG-I like receptors and their signaling crosstalk in the regulation of antiviral immunity', *Current Opinion in Virology*, 1(3), p. 167. doi: 10.1016/J.COVIRO.2011.04.004.

Rampanelli, E. *et al.* (2018) 'Excessive dietary lipid intake provokes an acquired form of lysosomal lipid storage disease in the kidney', 246(4), pp. 470–484. doi: 10.1002/PATH.5150.

Ranjan, P. *et al.* (2009) 'Cytoplasmic nucleic acid sensors in antiviral immunity', *Trends in Molecular Medicine*, 15(8), pp. 359–368. doi: 10.1016/J.MOLMED.2009.06.003/ASSET/CE0FFFAB-FC9C-42FC-9C18-2879C8BA8DE0/MAIN.ASSETS/GR3.JPG.

Ratajczak, M. Z. and Kucia, M. (2021) 'Hematopoiesis and innate immunity: an inseparable couple for good and bad times, bound together by an hormetic relationship', *Leukemia 2021 36:1*, 36(1), pp. 23–32. doi: 10.1038/s41375-021-01482-0.

Rathinam, V. A. K. *et al.* (2010) 'The AIM2 inflammasome is essential for host defense against cytosolic bacteria and DNA viruses', 11(5), pp. 395–402. doi: 10.1038/ni.1864.

Rathinam, V. A. K. *et al.* (2012) 'TRIF licenses caspase-11-dependent NLRP3 inflammasome activation by gram-negative bacteria', *Cell*, 150(3), pp. 606–619. doi: 10.1016/J.CELL.2012.07.007.

Reaves, B. and Banting, G. (1994) 'Vacuolar ATPase inactivation blocks recycling to the trans-Golgi network from the plasma membrane', *FEBS letters*, 345(1), pp. 61–66. doi: 10.1016/0014-5793(94)00437-4.

Rehwinkel, J. and Gack, M. U. (2020) 'RIG-I-like receptors: their regulation and roles in RNA sensing', *Nature Reviews Immunology 2020 20:9*, 20(9), pp. 537–551. doi: 10.1038/s41577-020-0288-3.

Ren, K. and Torres, R. (2008) 'Role of interleukin-1β during pain and inflammation', *Brain research reviews*, 60(1), p. 57. doi: 10.1016/J.BRAINRESREV.2008.12.020.

Riedl, S. J. and Salvesen, G. S. (2007) 'The apoptosome: signalling platform of cell death', *Nature reviews. Molecular cell biology*, 8(5), pp. 405–413. doi: 10.1038/NRM2153.

Roberts, T. L. *et al.* (2009) 'HIN-200 proteins regulate caspase activation in response

to foreign cytoplasmic DNA', *Science*. doi: 10.1126/science.1169841.

Roden, M. (2006) 'Mechanisms of Disease: hepatic steatosis in type 2 diabetes—pathogenesis and clinical relevance', *Nature Clinical Practice Endocrinology & Metabolism 2006 2:6*, 2(6), pp. 335–348. doi: 10.1038/ncpendmet0190.

Rogers, C. *et al.* (2019) 'Gasdermin pores permeabilize mitochondria to augment caspase-3 activation during apoptosis and inflammasome activation', *Nature communications*, 10(1). doi: 10.1038/S41467-019-09397-2.

Romani, N. *et al.* (1994) 'Proliferating dendritic cell progenitors in human blood.', *Journal of Experimental Medicine*, 180(1), pp. 83–93. doi: 10.1084/JEM.180.1.83.

Romberg, N., Vogel, T. P. and Canna, S. W. (2017a) 'NLRC4 inflammasomopathies', *Current opinion in allergy and clinical immunology*, 17(6), pp. 398–404. doi: 10.1097/ACI.0000000000000396.

Romberg, N., Vogel, T. P. and Canna, S. W. (2017b) 'NLRC4 inflammasomopathies', *Current opinion in allergy and clinical immunology*, 17(6), p. 398. doi: 10.1097/ACI.0000000000000396.

Rooney, S. A. (1989) 'Fatty acid biosynthesis in developing fetal lung', *The American journal of physiology*, 257(4 Pt 1). doi: 10.1152/AJPLUNG.1989.257.4.L195.

Rosenberg, G. *et al.* (2021) 'Host succinate is an activation signal for Salmonella virulence during intracellular infection', *Science*, 371(6527), pp. 400–405. doi: 10.1126/SCIENCE.ABA8026/SUPPL_FILE/ABA8026_TABLE_S9.XLSX.

Rysman, E. *et al.* (2010) 'De novo lipogenesis protects cancer cells from free radicals and chemotherapeutics by promoting membrane lipid saturation', *Cancer research*, 70(20), pp. 8117–8126. doi: 10.1158/0008-5472.CAN-09-3871.

Saleem, M. A., Mueller, A. and Bhattacharya, S. K. (2023) 'Lipidomics Analysis with Triple Quadrupole Mass Spectrometry', *Methods in molecular biology (Clifton, N.J.)*, 2625, pp. 103–106. doi: 10.1007/978-1-0716-2966-6_9.

Salihovic, S. *et al.* (2023) 'Recent advances towards mass spectrometry-based clinical lipidomics', *Current Opinion in Chemical Biology*, 76, p. 102370. doi: 10.1016/J.CBPA.2023.102370.

Samovski, D. *et al.* (2015) 'Regulation of AMPK activation by CD36 links fatty acid uptake to β-oxidation', *Diabetes*, 64(2), pp. 353–359. doi: 10.2337/DB14-0582.

Sanman, L. E. *et al.* (2016) 'Disruption of glycolytic flux is a signal for inflammasome signaling and pyroptotic cell death', *eLife*, 5(MARCH2016). doi: 10.7554/ELIFE.13663.

Sano, M. *et al.* (2020) 'Palmitic acid activates NLRP3 inflammasome and induces placental inflammation during pregnancy in mice', *Journal of Reproduction and Development*, 66(3), pp. 241–248. doi: 10.1262/JRD.2020-007.

Santos, J. C. *et al.* (2020) 'Human GBP1 binds LPS to initiate assembly of a caspase-4 activating platform on cytosolic bacteria', *Nature Communications 2020 11:1*, 11(1), pp. 1–15. doi: 10.1038/s41467-020-16889-z.

Saponaro, C. *et al.* (2015) 'The Subtle Balance between Lipolysis and Lipogenesis:

A Critical Point in Metabolic Homeostasis', *Nutrients*, 7(11), p. 9453. doi: 10.3390/NU7115475.

Sborgi, L. *et al.* (2016) 'GSDMD membrane pore formation constitutes the mechanism of pyroptotic cell death', *The EMBO journal*, 35(16), pp. 1766–1778. doi: 10.15252/EMBJ.201694696.

Schmid-Burgk, J. L. *et al.* (2016) 'A Genome-wide CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) Screen Identifies NEK7 as an Essential Component of NLRP3 Inflammasome Activation', *Journal of Biological Chemistry*, 291(1), pp. 103–109. doi: 10.1074/JBC.C115.700492.

Schorn, C. *et al.* (2011) 'Sodium overload and water influx activate the NALP3 inflammasome', *The Journal of biological chemistry*, 286(1), pp. 35–41. doi: 10.1074/JBC.M110.139048.

Schroder, K. and Tschopp, J. (2010) 'The inflammasomes', *Cell*, 140(6), pp. 821–832. doi: 10.1016/J.CELL.2010.01.040.

Schumann, J. (2016) 'It is all about fluidity: Fatty acids and macrophage phagocytosis', *European journal of pharmacology*, 785, pp. 18–23. doi: 10.1016/J.EJPHAR.2015.04.057.

Shao, L. *et al.* (2020) 'SUMO1 SUMOylates and SENP3 deSUMOylates NLRP3 to orchestrate the inflammasome activation', *FASEB Journal*, 34(1), pp. 1497–1515. doi: 10.1096/FJ.201901653R.

Sharif, H. *et al.* (2019) 'Structural mechanism for NEK7-licensed activation of NLRP3 inflammasome', *Nature 2019 570:7761*, 570(7761), pp. 338–343. doi: 10.1038/s41586-019-1295-z.

Sharma, M. and de Alba, E. (2023) 'Assembly mechanism of the inflammasome sensor AIM2 revealed by single molecule analysis', *Nature Communications 2023 14:1*, 14(1), pp. 1–16. doi: 10.1038/s41467-023-43691-4.

Sheikh, S. *et al.* (2005) 'Differing mechanisms of leukocyte recruitment and sensitivity to conditioning by shear stress for endothelial cells treated with tumour necrosis factor-α or interleukin-1β', *British Journal of Pharmacology*, 145(8), p. 1052. doi: 10.1038/SJ.BJP.0706281.

Sherwood, E. R. *et al.* (2022) 'Innate Immune Memory and the Host Response to Infection', *Journal of immunology (Baltimore, Md. : 1950)*, 208(4), pp. 785–792. Available at: www.jimmunol.org/cgi/doi/10.4049/jimmunol.2101058 (Accessed: 16 January 2024).

Shi, H. *et al.* 'NLRP3 activation and mitosis are mutually exclusive events coordinated by NEK7, a new inflammasome component', 17(3), pp. 250–258. doi: 10.1038/ni.3333.

Shi, H. *et al.* (2006) 'TLR4 links innate immunity and fatty acid-induced insulin resistance', *The Journal of clinical investigation*, 116(11), pp. 3015–3025. doi: 10.1172/JCI28898.

Shi, H. *et al.* (2016) 'NLRP3 activation and mitosis are mutually exclusive events coordinated by NEK7, a new inflammasome component', *Nature immunology*, 17(3), pp. 250–258. doi: 10.1038/NI.3333.

Shi, H., Murray, A. and Beutler, B. (2016) ‘Reconstruction of the Mouse Inflammasome System in HEK293T Cells’, *Bio-protocol*, 6(21). doi: 10.21769/BIOPROTOC.1986.

Shi, J. *et al.* (2014) ‘Inflammatory caspases are innate immune receptors for intracellular LPS’, *Nature*, 514(7521), pp. 187–192. doi: 10.1038/NATURE13683.

Shi, J. *et al.* (2015) ‘Cleavage of GSDMD by inflammatory caspases determines pyroptotic cell death’, 526(7575), pp. 660–665. Available at: https://www.nature.com/articles/nature15514 (Accessed: 13 March 2023).

Shim, D. W. *et al.* (2017) ‘BOT-4-one attenuates NLRP3 inflammasome activation: NLRP3 alkylation leading to the regulation of its ATPase activity and ubiquitination’, *Scientific Reports 2017 7:1*, 7(1), pp. 1–12. doi: 10.1038/s41598-017-15314-8.

Shimano, H. and Sato, R. (2017) ‘SREBP-regulated lipid metabolism: convergent physiology — divergent pathophysiology’, *Nature Reviews Endocrinology 2017 13:12*, 13(12), pp. 710–730. doi: 10.1038/nrendo.2017.91.

Shimizu, T. (2009) ‘Lipid mediators in health and disease: enzymes and receptors as therapeutic targets for the regulation of immunity and inflammation’, *Annual review of pharmacology and toxicology*, 49, pp. 123–150. doi: 10.1146/ANNUREV.PHARMTOX.011008.145616.

Shirasuna, K. *et al.* (2016) ‘Palmitic acid induces interleukin-1β secretion via NLRP3 inflammasomes and inflammatory responses through ROS production in human placental cells’, *Journal of Reproductive Immunology*, 116, pp. 104–112. doi: 10.1016/J.JRI.2016.06.001.

Singh, P. *et al.* (2013) ‘Cholesterol Biosynthesis and Homeostasis in Regulation of the Cell Cycle’, *PLoS ONE*, 8(3), p. 58833. doi: 10.1371/JOURNAL.PONE.0058833.

Sokolowska, M. *et al.* (2015) ‘Prostaglandin E2 inhibits NLRP3 inflammasome activation through EP4 receptor and intracellular cAMP in human macrophages’, *Journal of immunology (Baltimore, Md. : 1950)*, 194(11), p. 5472. doi: 10.4049/JIMMUNOL.1401343.

Song, H. *et al.* (2016) ‘The E3 ubiquitin ligase TRIM31 attenuates NLRP3 inflammasome activation by promoting proteasomal degradation of NLRP3’, 7(1), pp. 1–11. Available at: https://pubmed.ncbi.nlm.nih.gov/27929086/ (Accessed: 6 October 2024).

Song, N. *et al.* (2017) ‘NLRP3 Phosphorylation Is an Essential Priming Event for Inflammasome Activation’, *Molecular cell*, 68(1), pp. 185-197.e6. doi: 10.1016/J.MOLCEL.2017.08.017.

Song, N. and Li, T. (2018) ‘Regulation of NLRP3 inflammasome by phosphorylation’, *Frontiers in Immunology*, 9(OCT), p. 411448. doi: 10.3389/FIMMU.2018.02305/BIBTEX.

Souza, A. L. and Patti, G. J. (2021) ‘A Protocol for Untargeted Metabolomic Analysis: From Sample Preparation to Data Processing’, *Methods in molecular biology (Clifton, N.J.)*, 2276, p. 357. doi: 10.1007/978-1-0716-1266-8_27.

Stadnyk, A. W. (1994) ‘Cytokine production by epithelial cells’, *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*,

8(13), pp. 1041–1047. doi: 10.1096/FASEBJ.8.13.7926369.

Stella Ademowo, O., Wenk, M. R. and Maier, A. B. (2024) ‘Advances in clinical application of lipidomics in healthy ageing and healthy longevity medicine’. doi: 10.1016/j.arr.2024.102432.

Stone, K. D., Prussin, C. and Metcalfe, D. D. (2010) ‘IgE, Mast Cells, Basophils, and Eosinophils’, *The Journal of allergy and clinical immunology*, 125(2 Suppl 2), p. S73. doi: 10.1016/J.JACI.2009.11.017.

Strizova, Z. *et al.* (2023) ‘M1/M2 macrophages and their overlaps – myth or reality?’, *Clinical Science (London, England : 1979)*, 137(15), p. 1067. doi: 10.1042/CS20220531.

Stutz, A. *et al.* (2017) ‘NLRP3 inflammasome assembly is regulated by phosphorylation of the pyrin domain’, *The Journal of Experimental Medicine*, 214(6), p. 1725. doi: 10.1084/JEM.20160933.

Subramanian, N. *et al.* (2013) ‘The adaptor MAVS promotes NLRP3 mitochondrial localization and inflammasome activation’, *Cell*, 153(2), pp. 348–361. doi: 10.1016/J.CELL.2013.02.054/ATTACHMENT/60326331-EC13-43BE-828E-3568FB9CCCDB/MMC5.PDF.

Summers, C. *et al.* (2010) ‘Neutrophil kinetics in health and disease’, *Trends in Immunology*, 31(8), p. 318. doi: 10.1016/J.IT.2010.05.006.

Sun, J. C. and Lanier, L. L. (2009) ‘Natural killer cells remember: an evolutionary bridge between innate and adaptive immunity?’, *European journal of immunology*, 39(8), pp. 2059–2064. doi: 10.1002/EJI.200939435.

Sun, M. and Zhang, X. (2022) ‘Current methodologies in protein ubiquitination characterization: from ubiquitinated protein to ubiquitin chain architecture’, *Cell & Bioscience 2022 12:1*, 12(1), pp. 1–17. doi: 10.1186/S13578-022-00870-Y.

Sun, X. *et al.* (2022) ‘Macrophage Polarization, Metabolic Reprogramming, and Inflammatory Effects in Ischemic Heart Disease’, *Frontiers in Immunology*, 13, p. 934040. doi: 10.3389/FIMMU.2022.934040/BIBTEX.

Sundaram, B. and Kanneganti, T. D. (2021) ‘Advances in Understanding Activation and Function of the NLRC4 Inflammasome’, *International Journal of Molecular Sciences*, 22(3), pp. 1–12. doi: 10.3390/IJMS22031048.

Sunshine, H. and Iruela-Arispe, M. L. (2017) ‘Membrane lipids and cell signaling’, *Current Opinion in Lipidology*, pp. 408–413. doi: 10.1097/MOL.0000000000000443.

Swanson, K. V., Deng, M. and Ting, J. P. Y. (2019) *The NLRP3 inflammasome: molecular activation and regulation to therapeutics*. Nature Publishing Group. Available at: https://pmc.ncbi.nlm.nih.gov/articles/PMC7807242/ (Accessed: 28 April 2021).

Swanton, T. *et al.* (2020) ‘Selective inhibition of the K+ efflux sensitive NLRP3 pathway by Cl− channel modulation’, *Chemical Science*, 11(43), pp. 11720–11728. doi: 10.1039/D0SC03828H.

Swinnen, J. V. *et al.* (2003) ‘Fatty acid synthase drives the synthesis of phospholipids partitioning into detergent-resistant membrane microdomains’,

Biochemical and Biophysical Research Communications, 302(4), pp. 898–903. doi: 10.1016/S0006-291X(03)00265-1.

Tabaczar, S. *et al.* (2017) 'Protein palmitoylation: Palmitoyltransferases and their specificity', *Experimental Biology and Medicine*, 242(11), p. 1150. doi: 10.1177/1535370217707732.

Tabeta, K. *et al.* (2004) 'Toll-like receptors 9 and 3 as essential complonents of innate immune defense against mouse cytomegalovirus infection', *Proceedings of the National Academy of Sciences of the United States of America*, 101(10), pp. 3516–3521. doi: 10.1073/pnas.0400525101.

Takáts, Z. *et al.* (2004) 'Mass spectrometry sampling under ambient conditions with desorption electrospray ionization', *Science (New York, N.Y.)*, 306(5695), pp. 471–473. doi: 10.1126/SCIENCE.1104404.

Tall, A. R. and Yvan-Charvet, L. (2015) *Cholesterol, inflammation and innate immunity*. Available at: /pmc/articles/PMC4669071/ (Accessed: 10 May 2021).

Tannahill, G. M. *et al.* (2013) 'Succinate is an inflammatory signal that induces IL-1β through HIF-1α', *Nature 2013 496:7444*, 496(7444), pp. 238–242. doi: 10.1038/nature11986.

Theilgaard-Mönch, K. *et al.* (2004) 'The transcriptional activation program of human neutrophils in skin lesions supports their important role in wound healing', *Journal of immunology (Baltimore, Md. : 1950)*, 172(12), pp. 7684–7693. doi: 10.4049/JIMMUNOL.172.12.7684.

Ting, J. P. Y. *et al.* (2008) 'The NLR gene family: a standard nomenclature', *Immunity*, 28(3), pp. 285–287. doi: 10.1016/J.IMMUNI.2008.02.005.

Tipton, D. A., Pabst, M. J. and Dabbous, M. K. (1990) 'Interleukin-1β- and tumor necrosis factor-α-independent monocyte stimulation of fibroblast collagenase activity', *Journal of Cellular Biochemistry*, 44(4), pp. 253–264. doi: 10.1002/jcb.240440407.

Traven, A. and Naderer, T. (2019) 'Central metabolic interactions of immune cells and microbes: prospects for defeating infections', *EMBO reports*, 20(7). doi: 10.15252/EMBR.201947995.

Trouw, L. A. and Daha, M. R. (2011) 'Role of complement in innate immunity and host defense', *Immunology letters*, 138(1), pp. 35–37. doi: 10.1016/J.IMLET.2011.02.014.

Trub, A. G. *et al.* (2022a) 'Statin therapy inhibits fatty acid synthase via dynamic protein modifications', *Nature Communications 2022 13:1*, 13(1), pp. 1–14. doi: 10.1038/s41467-022-30060-w.

Trub, A. G. *et al.* (2022b) 'Statin therapy inhibits fatty acid synthase via dynamic protein modifications', *Nature Communications*, 13(1), pp. 1–14. doi: 10.1038/s41467-022-30060-w.

Tyrtyshnaia, A. *et al.* (2022) 'Fatty Acid-Derived N-acylethanolamines Dietary Supplementation Attenuates Neuroinflammation and Cognitive Impairment in LPS Murine Model', *Nutrients*, 14(18). doi: 10.3390/NU14183879/S1.

Ullevig, S. L. *et al.* (2016) 'Protein S-Glutathionylation Mediates Macrophage Responses to Metabolic Cues from the Extracellular Environment', *Antioxidants & redox signaling*, 25(15), pp. 836–851. doi: 10.1089/ARS.2015.6531.

Vanaja, S. K. *et al.* (2016) 'Bacterial Outer Membrane Vesicles Mediate Cytosolic Localization of LPS and Caspase-11 Activation', *Cell*, 165(5), pp. 1106–1119. doi: 10.1016/J.CELL.2016.04.015.

Vance, J. E. and Tasseva, G. (2013) 'Formation and function of phosphatidylserine and phosphatidylethanolamine in mammalian cells', *Biochimica et biophysica acta*, 1831(3), pp. 543–554. doi: 10.1016/J.BBALIP.2012.08.016.

Vandanmagsar, B. *et al.* (2011) 'The NALP3/NLRP3 Inflammasome Instigates Obesity-Induced Autoinflammation and Insulin Resistance', *Nature medicine*, 17(2), p. 179. doi: 10.1038/NM.2279.

van der Veen, J. N. *et al.* (2017) 'The critical role of phosphatidylcholine and phosphatidylethanolamine metabolism in health and disease', *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 1859(9), pp. 1558–1572. doi: 10.1016/J.BBAMEM.2017.04.006.

Verhoef, P. A. *et al.* (2005) 'Inhibitory effects of chloride on the activation of caspase-1, IL-1beta secretion, and cytolysis by the P2X7 receptor', *Journal of immunology (Baltimore, Md. : 1950)*, 175(11), pp. 7623–7634. doi: 10.4049/JIMMUNOL.175.11.7623.

Vidali, G., Gershey, E. L. and Allfrey, V. G. (1968) 'Chemical Studies of Histone Acetylation', *Journal of Biological Chemistry*, 243(24), pp. 6361–6366. doi: 10.1016/s0021-9258(18)93148-0.

Vidya, M. K. *et al.* (2018) *Toll-like receptors: Significance, ligands, signaling pathways, and functions in mammals*. Int Rev Immunol. doi: 10.1080/08830185.2017.1380200.

Viganò, E. *et al.* (2015) 'Human caspase-4 and caspase-5 regulate the one-step non-canonical inflammasome activation in monocytes', *Nature Communications 2015 6:1*, 6(1), pp. 1–13. doi: 10.1038/ncomms9761.

Villar, J. *et al.* (2023) 'Monocytes differentiate along two alternative pathways during sterile inflammation', *EMBO reports*, 24(7). doi: 10.15252/EMBR.202256308/SUPPL_FILE/EMBR202256308-SUP-0003-DATASETEV2.XLSX.

Vivier, E. *et al.* (2008) 'Functions of natural killer cells', *Nature Immunology 2008 9:5*, 9(5), pp. 503–510. doi: 10.1038/ni1582.

Vorobjeva, N. V. and Chernyak, B. V. (2020) 'NETosis: Molecular Mechanisms, Role in Physiology and Pathology', *Biochemistry. Biokhimiia*, 85(10), p. 1178. doi: 10.1134/S0006297920100065.

Voskoboinik, I., Whisstock, J. C. and Trapani, J. A. (2015) 'Perforin and granzymes: function, dysfunction and human pathology', *Nature Reviews Immunology 2015 15:6*, 15(6), pp. 388–400. doi: 10.1038/nri3839.

Wager, C. M. L. and Wormley, F. L. (2014) 'Classical versus alternative macrophage activation: the Ying and the Yang in host defense against pulmonary fungal

infections', *Mucosal Immunology 2014 7:5*, 7(5), pp. 1023–1035. doi: 10.1038/mi.2014.65.

Wakil, S. J., Stoops, J. K. and Joshi, V. C. (1983) 'Fatty acid synthesis and its regulation', (Volume 52, 1983), pp. 537–579. Available at: https://pubmed.ncbi.nlm.nih.gov/6137188/ (Accessed: 23 September 2024).

Waldhuber, A. *et al.* (2016) 'Uropathogenic Escherichia coli strain CFT073 disrupts NLRP3 inflammasome activation', *The Journal of clinical investigation*, 126(7), pp. 2425–2436. doi: 10.1172/JCI81916.

Walev, I. *et al.* (1995) 'Potassium-inhibited processing of IL-1 beta in human monocytes', *The EMBO Journal*, 14(8), p. 1607. doi: 10.1002/J.1460-2075.1995.TB07149.X.

Wandel, M. P. *et al.* (2020) 'Guanylate-Binding Proteins (GBPs) convert cytosolic bacteria into caspase-4 signaling platforms', *Nature immunology*, 21(8), p. 880. doi: 10.1038/S41590-020-0697-2.

Wang, B. and Yin, Q. (2017) 'AIM2 inflammasome activation and regulation: A structural perspective', *Journal of structural biology*, 200(3), p. 279. doi: 10.1016/J.JSB.2017.08.001.

Wang, J. *et al.* (2020) 'High glucose mediates NLRP3 inflammasome activation via upregulation of ELF3 expression', *Cell death & disease*, 11(5). doi: 10.1038/S41419-020-2598-6.

Wang, L. *et al.* (2023) 'Palmitoylation prevents sustained inflammation by limiting NLRP3 inflammasome activation through chaperone-mediated autophagy', 83(2), pp. 281-297.e10. doi: 10.1016/J.MOLCEL.2022.12.002.

Wang, S. *et al.* (2010) 'AMPKalpha2 deletion causes aberrant expression and activation of NAD(P)H oxidase and consequent endothelial dysfunction in vivo: role of 26S proteasomes', *Circulation research*, 106(6), pp. 1117–1128. doi: 10.1161/CIRCRESAHA.109.212530.

Wang, Y. and Huang, S. (2017) 'Golgi structure formation, function, and post-translational modifications in mammalian cells', *F1000Research*, 6. doi: 10.12688/F1000RESEARCH.11900.1.

Warren, S. E. *et al.* (2010) 'Cutting edge: Cytosolic bacterial DNA activates the inflammasome via Aim2', *Journal of immunology (Baltimore, Md. : 1950)*, 185(2), pp. 818–821. doi: 10.4049/JIMMUNOL.1000724.

Wculek, S. K. *et al.* (2019) 'Dendritic cells in cancer immunology and immunotherapy', *Nature Reviews Immunology 2019 20:1*, 20(1), pp. 7–24. doi: 10.1038/s41577-019-0210-z.

Wei, X. *et al.* (2016) 'Fatty acid synthesis configures the plasma membrane for inflammation in diabetes', *Nature*, 539(7628). Available at: /pmc/articles/PMC5671339/ (Accessed: 10 May 2021).

Weiskopf, K. *et al.* (2016) 'Myeloid cell origins, differentiation, and clinical implications', *Microbiology spectrum*, 4(5), pp. 10.1128/microbiolspec.MCHD-0031–2016. doi: 10.1128/MICROBIOLSPEC.MCHD-0031-2016.

Wen, H. *et al.* (2011a) ‘Fatty acid–induced NLRP3-ASC inflammasome activation interferes with insulin signaling’, *Nature Immunology 2011 12:5*, 12(5), pp. 408–415. doi: 10.1038/ni.2022.

Wen, H. *et al.* (2011b) ‘Fatty acid–induced NLRP3-ASC inflammasome activation interferes with insulin signaling’, *Nature Immunology 2011 12:5*, 12(5), pp. 408–415. doi: 10.1038/ni.2022.

Wen, J. *et al.* (2021) ‘Updating the NLRC4 Inflammasome: from Bacterial Infections to Autoimmunity and Cancer’, *Frontiers in Immunology*, 12. doi: 10.3389/FIMMU.2021.702527.

Wenk, M. R. (2005) ‘The emerging field of lipidomics’, *Nature reviews. Drug discovery*, 4(7), pp. 594–610. doi: 10.1038/NRD1776.

West, E. E. and Kemper, C. (2023) ‘Complosome - the intracellular complement system’, *Nature reviews. Nephrology*, 19(7), pp. 426–439. doi: 10.1038/S41581-023-00704-1.

Williams, N. C. and O’Neill, L. A. J. (2018) ‘A Role for the Krebs Cycle Intermediate Citrate in Metabolic Reprogramming in Innate Immunity and Inflammation’, *Frontiers in immunology*, 9(FEB). doi: 10.3389/FIMMU.2018.00141.

Wolf, A. J. *et al.* (2016) ‘Hexokinase Is an Innate Immune Receptor for the Detection of Bacterial Peptidoglycan’, *Cell*, 166(3), pp. 624–636. doi: 10.1016/J.CELL.2016.05.076/ATTACHMENT/2F6DD9E3-B81D-4849-B1DD-D2ABC3BC909E/MMC2.PDF.

Wymann, M. P. and Schneiter, R. (2008a) ‘Lipid signalling in disease’, *Nature Reviews Molecular Cell Biology 2008 9:2*, 9(2), pp. 162–176. doi: 10.1038/nrm2335.

Wymann, M. P. and Schneiter, R. (2008b) ‘Lipid signalling in disease’, *Nature Reviews Molecular Cell Biology 2008 9:2*, 9(2), pp. 162–176. doi: 10.1038/nrm2335.

Xia, J. *et al.* (2023) ‘The Role of Post-Translational Modifications in Regulation of NLRP3 Inflammasome Activation’, *International Journal of Molecular Sciences*, 24(7), p. 24. doi: 10.3390/IJMS24076126.

Xia, S. *et al.* (2021) ‘Gasdermin D pore structure reveals preferential release of mature interleukin-1’, *Nature*, 593(7860), p. 607. doi: 10.1038/S41586-021-03478-3.

Xian, H. *et al.* (2022) ‘Oxidized DNA fragments exit mitochondria via mPTP- and VDAC-dependent channels to activate NLRP3 inflammasome and interferon signaling’, *Immunity*, 55(8), pp. 1370-1385.e8. doi: 10.1016/J.IMMUNI.2022.06.007.

Xiao, Y. *et al.* (2024) ‘The implications of FASN in immune cell biology and related diseases’, *Cell Death & Disease 2024 15:1*, 15(1), pp. 1–12. doi: 10.1038/s41419-024-06463-6.

Xiong, W. *et al.* (2021) ‘Metformin alleviates inflammation through suppressing FASN-dependent palmitoylation of Akt’, *Cell Death & Disease 2021 12:10*, 12(10), pp. 1–15. doi: 10.1038/s41419-021-04235-0.

Xu, S. *et al.* (2015) ‘Palmitate induces ER calcium depletion and apoptosis in mouse podocytes subsequent to mitochondrial oxidative stress’, *Cell Death & Disease 2015 6:11*, 6(11), pp. e1976–e1976. doi: 10.1038/cddis.2015.331.

Xu, T. *et al.* (2022) ‘Ubiquitination of NLRP3 by gp78/Insig-1 restrains NLRP3 inflammasome activation’, *Cell Death & Differentiation 2022 29:8*, 29(8), pp. 1582–1595. doi: 10.1038/s41418-022-00947-8.

Xu, Z. *et al.* (2020) ‘Distinct Molecular Mechanisms Underlying Potassium Efflux for NLRP3 Inflammasome Activation’, *Frontiers in Immunology*, 11. doi: 10.3389/FIMMU.2020.609441.

Xu, Z. *et al.* (2021) ‘NLRP3 inflammasome promoted the malignant progression of prostate cancer via the activation of caspase-1’, *Cell Death Discovery 2021 7:1*, 7(1), pp. 1–9. doi: 10.1038/s41420-021-00766-9.

Yang, H. C. *et al.* (2019) ‘The Redox Role of G6PD in Cell Growth, Cell Death, and Cancer’, *Cells*, 8(9). doi: 10.3390/CELLS8091055.

Yang, J. *et al.* (2013) ‘Human NAIP and mouse NAIP1 recognize bacterial type III secretion needle protein for inflammasome activation’, *Proceedings of the National Academy of Sciences*. doi: 10.1073/pnas.1306376110.

Yang, M. *et al.* (2021) ‘AIM2 deficiency in B cells ameliorates systemic lupus erythematosus by regulating Blimp-1–Bcl-6 axis-mediated B-cell differentiation’, *Signal Transduction and Targeted Therapy 2021 6:1*, 6(1), pp. 1–11. doi: 10.1038/s41392-021-00725-x.

Yang, T. *et al.* (2002) ‘Crucial Step in Cholesterol Homeostasis: Sterols Promote Binding of SCAP to INSIG-1, a Membrane Protein that Facilitates Retention of SREBPs in ER’, *Cell*, 110(4), pp. 489–500. doi: 10.1016/S0092-8674(02)00872-3.

Yang, Y. *et al.* (2019) ‘Recent advances in the mechanisms of NLRP3 inflammasome activation and its inhibitors’, *Cell Death and Disease*. Nature Publishing Group, pp. 1–11. doi: 10.1038/s41419-019-1413-8.

Ye, J. and DeBose-Boyd, R. A. (2011) ‘Regulation of cholesterol and fatty acid synthesis’, *Cold Spring Harbor Perspectives in Biology*, pp. 1–13. doi: 10.1101/cshperspect.a004754.

Yen, W. C. *et al.* (2019) ‘Impaired inflammasome activation and bacterial clearance in G6PD deficiency due to defective NOX/p38 MAPK/AP-1 redox signaling’, *Redox Biology*, 28, p. 101363. doi: 10.1016/J.REDOX.2019.101363.

Yu, X. *et al.* (2024) ‘Structural basis for the oligomerization-facilitated NLRP3 activation’, *Nature Communications 2024 15:1*, 15(1), pp. 1–13. doi: 10.1038/s41467-024-45396-8.

Yu, Y. R. A. *et al.* (2016) ‘Flow cytometric analysis of myeloid cells in human blood, bronchoalveolar lavage, and lung tissues’, *American Journal of Respiratory Cell and Molecular Biology*, 54(1), pp. 13–24. doi: 10.1165/RCMB.2015-0146OC/SUPPL_FILE/DISCLOSURES.PDF.

Zadoorian, A., Du, X. and Yang, H. (2023) ‘Lipid droplet biogenesis and functions in health and disease’, *Nature Reviews Endocrinology 2023 19:8*, 19(8), pp. 443–459. doi: 10.1038/s41574-023-00845-0.

Zandieh, Z. *et al.* (2023) ‘TLR-1, TLR-2, and TLR-6 MYD88-dependent signaling pathway: A potential factor in the interaction of high-DNA fragmentation human sperm with fallopian tube epithelial cells’, *Clinical and experimental reproductive*

medicine, 50(1), pp. 44–52. doi: 10.5653/CERM.2022.05610.

Zanoni, I. *et al.* (2016) 'An endogenous caspase-11 ligand elicits interleukin-1 release from living dendritic cells', 352(6290). Available at: /pmc/articles/PMC5111085/ (Accessed: 23 May 2024).

Zasłona, Z. *et al.* (2020) 'Caspase-11 promotes allergic airway inflammation', *Nature Communications 2020 11:1*, 11(1), pp. 1–11. doi: 10.1038/s41467-020-14945-2.

Zhang. *et al.* (2023) 'Distinct changes in endosomal composition promote NLRP3 inflammasome activation', 24(1). doi: 10.1038/S41590-022-01355-3.

Zhang, H. and Forman, H. J. (2012) 'Glutathione synthesis and its role in redox signaling', *Seminars in Cell & Developmental Biology*, 23(7), pp. 722–728. doi: 10.1016/J.SEMCDB.2012.03.017.

Zhang, L. *et al.* (2018) 'POH1 deubiquitinates pro-interleukin-1β and restricts inflammasome activity', *Nature communications*, 9(1). doi: 10.1038/S41467-018-06455-Z.

Zhang, N. *et al.* (2024) 'A palmitoylation–depalmitoylation relay spatiotemporally controls GSDMD activation in pyroptosis', *Nature Cell Biology 2024 26:5*, 26(5), pp. 757–769. doi: 10.1038/s41556-024-01397-9.

Zhang, R. *et al.* (2023) 'Histone malonylation is regulated by SIRT5 and KAT2A', *iScience*, 26(3), p. 106193. doi: 10.1016/J.ISCI.2023.106193.

Zhang, W. *et al.* (2020) 'Inhibition of fatty acid synthase (FASN) affects the proliferation and apoptosis of HepG2 hepatoma carcinoma cells via the β-catenin/C-myc signaling pathway', *Annals of Hepatology*, 19(4), pp. 411–416. doi: 10.1016/J.AOHEP.2020.03.005.

Zhang, Y. *et al.* (2013) 'Point mutation in luminal loop 7 of Scap protein blocks interaction with loop 1 and abolishes movement to Golgi', *The Journal of biological chemistry*, 288(20), pp. 14059–14067. doi: 10.1074/JBC.M113.469528.

Zhang, Y. *et al.* (2018) 'Plasma membrane changes during programmed cell deaths', *Cell Res.*, 28(1), pp. 9–21. doi: 10.1038/cr.2017.133.

Zhang, Y. *et al.* (2019) 'Melatonin modulates IL-1β-induced extracellular matrix remodeling in human nucleus pulposus cells and attenuates rat intervertebral disc degeneration and inflammation', *Aging (Albany NY)*, 11(22), p. 10499. doi: 10.18632/AGING.102472.

Zhang, Y. *et al.* (2021) 'Function of Protein S-Palmitoylation in Immunity and Immune-Related Diseases', *Frontiers in Immunology*, 12, p. 661202. doi: 10.3389/FIMMU.2021.661202/BIBTEX.

Zhang, Yening *et al.* (2023) 'Acetylation is required for full activation of the NLRP3 inflammasome', *Nature Communications 2023 14:1*, 14(1), pp. 1–12. doi: 10.1038/s41467-023-44203-0.

Zhang, Yuxi *et al.* (2023) 'AIM2 and Psoriasis', *Frontiers in Immunology*, 14, p. 1085448. doi: 10.3389/FIMMU.2023.1085448/BIBTEX.

Zhang, Y. Z. *et al.* (2019) 'NLRP3 inflammasome and lipid metabolism analysis

based on UPLC-Q-TOF-MS in gouty nephropathy', *International Journal of Molecular Medicine*, 44(1), p. 172. doi: 10.3892/IJMM.2019.4176.

Zhang, Z. *et al.* (2017a) 'Protein kinase D at the Golgi controls NLRP3 inflammasome activation', 214(9), pp. 2671–2693. doi: 10.1084/JEM.20162040.

Zhang, Z. *et al.* (2017b) 'Protein kinase D at the Golgi controls NLRP3 inflammasome activation', *Journal of Experimental Medicine*, 214(9), pp. 2671–2693. doi: 10.1084/JEM.20162040.

Zhao, W. *et al.* (2020) 'AKT Regulates NLRP3 Inflammasome Activation by Phosphorylating NLRP3 Serine 5', *Journal of immunology (Baltimore, Md. : 1950)*, 205(8), pp. 2255–2264. doi: 10.4049/JIMMUNOL.2000649.

Zheng, D., Liwinski, T. and Elinav, E. (2020) 'Interaction between microbiota and immunity in health and disease', 30(6), pp. 492–506. doi: 10.1038/s41422-020-0332-7.

Zheng, S. *et al.* (2023) 'ZDHHC5-mediated NLRP3 palmitoylation promotes NLRP3-NEK7 interaction and inflammasome activation', 83(24), pp. 4570-4585.e7. doi: 10.1016/J.MOLCEL.2023.11.015.

Zhong, Z. *et al.* (2016) 'NF-κB Restricts inflammasome activation via elimination of damaged mitochondria', *Cell*, 164(5), p. 896. doi: 10.1016/J.CELL.2015.12.057.

Zhong, Z. *et al.* (2018a) 'New mitochondrial DNA synthesis enables NLRP3 inflammasome activation', *Nature 2018 560:7717*, 560(7717), pp. 198–203. doi: 10.1038/s41586-018-0372-z.

Zhong, Z. *et al.* (2018b) 'New mitochondrial DNA synthesis enables NLRP3 inflammasome activation', *Nature*, 560(7717), pp. 198–203. doi: 10.1038/S41586-018-0372-Z.

Zhou, Q. and Zhang, J. (2022) 'K27-linked noncanonic ubiquitination in immune regulation', *Journal of Leukocyte Biology*, 111(1), pp. 223–235. doi: 10.1002/JLB.4RU0620-397RR.

Zhou, R. *et al.* (2011) 'A role for mitochondria in NLRP3 inflammasome activation', *Nature*, 469(7329). Available at: https://pubmed.ncbi.nlm.nih.gov/21124315/ (Accessed: 28 July 2024).

Zhou, Y. *et al.* (2020) 'The Roles of Endoplasmic Reticulum in NLRP3 Inflammasome Activation', 9(5), p. 1219. doi: 10.3390/CELLS9051219.

Zhou, Z. *et al.* (2020) 'K63 ubiquitin chains target NLRP3 inflammasome for autophagic degradation in ox-LDL-stimulated THP-1 macrophages', *Aging (Albany NY)*, 12(2), p. 1747. doi: 10.18632/AGING.102710.

Zhu, K. *et al.* (2021) 'Priming of NLRP3 inflammasome activation by Msn kinase MINK1 in macrophages', *Cellular & Molecular Immunology 2021 18:10*, 18(10), pp. 2372–2382. doi: 10.1038/s41423-021-00761-1.

Zindel, J. and Kubes, P. (2020) 'DAMPs, PAMPs, and LAMPs in Immunity and Sterile Inflammation', *Annual Review of Pathology: Mechanisms of Disease*, 15, pp. 493–518. Available at: https://doi.org/10.1146/annurev-pathmechdis- (Accessed: 10 May 2024).

Zou, L. *et al.* (2023) ‘Lysine Malonylation and Its Links to Metabolism and Diseases’, *Aging and Disease*, 14(1), p. 84. doi: 10.14336/AD.2022.0711.